REMARKS

The Office Action mailed March 29, 2006 has been received and reviewed. The application is to be amended as previously set forth. Claims 1, 8, 9-11, and 16-20 have been amended. Claims 2-5, 13 and 21 are to be cancelled. No new matter has been entered. All of the pending claims 1-21 are presented below. Claims 12-21 were previously withdrawn. Claims 1-11 are currently under examination. Claims 1-7 and 9-11 stand rejected. Reconsideration is respectfully requested.

Interview summary

First, applicants wish to thank the Examiner and his supervisor for the courtesy extended applicants' representatives at the interview of May 15, 2006. Pursuant to MPEP § 713.04, applicants state the following:

Applicants' representatives discussed the rejections under 35 U.S.C. § 102 and § 103. Applicants' representatives explained why claims 1, 2, 4, 6 and 7 are novel over Ruben et al., and claims 1-7 are non-obvious over Ruben et al. in view of Fallaux et al. It appeared that the examiners followed the rationales of applicants' representatives', and agreed that the claims would overcome the rejections of record if claim 1 is amended to incorporate the limitations of claim 5, and incorporate the limitation that the cell has been obtained by stable transfection of the cell with the recombinant nucleic acid encoding the IgA molecule. Applicants' representatives also discussed the rejections under 35 U.S.C. § 112. Applicants agreed to cancel claims 2-5, and amend claims as previously set forth. It appeared in the interview that these cancellations and amendments would overcome the rejections under 35 U.S.C. § 112. With respect to the specification, applicants' representatives agreed to add the trademark symbol ™ for all occurrences of PER.C6 cells and add generic terminology to the first occurrence of PER.C6™.

Election/restrictions

Claims 12-21 were previously withdrawn from consideration. Applicants respectfully request rejoinder of the methods claims once the product claims have been allowed.

Information Disclosure Statement

Applicants thank the Examiner for retrieving the two documents listed in the Information Disclosure Statement and placing them in the file.

Specification Objection

The disclosure was objected to because of an informality. As discussed at the interview, the specification has been amended to include the trademark symbol with all occurrences of "PER.C6". Generic terminology, such as "human embryonic retinoblast cell line containing in its genome human adenovirus type 5 (Ad5) E1A and E1B coding sequences (nt. 459-3510) under the control of the human phosphoglycerate kinase (PGK) promoter", has been provided for PER.C6™ at its first occurrence, *i.e.* paragraph **[0016]** of the specification. To avoid a lengthy repeat of the generic terminology at every occurrence of PER.C6™, applicants believe that PER.C6™ itself should be sufficient, as also known to a person with ordinary skill in the art. Accordingly, applicants believe the amended specification is clear.

Claim Objections

Claims 9-11 were objected to because of an informality. Appropriate corrections have been made to the claims.

Claims 3-5 and 35 U.S.C. § 112, 2nd ¶

Claims 3-5 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite. It was thought that the recitation of "wherein said cell is derived from" renders claims 3-5 vague and indefinite. Claims 3-5 have been cancelled, therefore mooting the rejections as to them.

Claim 1 as amended incorporates the element of claim 5 and therefore also contains the term "wherein said cell is derived from", but it is submitted that in this claim it is fully clear what the steps involved in the derivation are, since the claim further specifies the derivation with the recitation "by stable transfection of said PER.C6™ cell with the recombinant nucleic acid encoding the IgA molecule in expressible format", which is believed to be fully clear and definite. Basis for the amendment can, for instance, be found in paragraph **[0035]** of the specification. Reconsideration is therefore respectfully requested.

Claim 5 and 35 U.S.C. § 112, 1st ¶

Claim 5 was rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement. It was thought that assurance of compliance with provisions of 37 C.F.R. §§ 1.801-1.809 in the form of a declaration or statement is required. A duly executed Budapest Treaty declaration and deposit receipt are enclosed herewith to overcome this rejection. The specification has been amended to include the deposit information. Withdrawal of the rejection is respectfully requested.

Claims 9-11 and 35 U.S.C. § 112, 1st ¶

Claims 9-11 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. It was suggested that the specification only provides examples with one cell and does not necessarily predict any other cells capable of achieving high production of IgA. It was suggested that Ruben *et al.* (U.S. Patent 6,475,753) (hereinafter "Ruben et al.") describes cells that express E1A and E1B proteins of an adenovirus and which comprise a recombinant nucleotide encoding an IgA molecule, but that Ruben et al. does not describe cells capable of producing at least 5, 20, or 40 pg IgA/seeded cell/day when seeded as directed in claims 9-11.

It is submitted that the limited structural variation in IgA molecules (mainly, variation will be possible within part of the variable region to accommodate antigen-specific binding, but further IgA molecules form a class of molecules that is believed to be structurally highly related, and thus so is the nucleic acid encoding them) makes it likely that similar results can be obtained for other than the exemplified IgA molecules, which other IgA molecules can, for instance, bind to different targets. In addition, claim 1 has been amended to incorporate the element that the cells are derived from PER.C6 cells and further has been directed to such cells stably transfected with the nucleic acid encoding the IgA molecule. It is submitted that it is clear that applicant at the time of filing had possession of the invention. Therefore, also in view of the amendment, reconsideration is respectfully requested.

Claim 1-7 and 9-11 and nonstatutory obviousness-type double patenting

Claims 1-7 and 9-11 were provisionally rejected on the grounds of nonstatutory obviousness-type double patenting as allegedly being unpatentable over claims 10-35, 41, and 42 of copending Application No. 10/234,007. Claims 1-7 and 9-11 were provisionally rejected on the grounds of nonstatutory obviousness-type double patenting as allegedly being unpatentable over claims 1-13, 17, and 18 of copending Application No. 10/790,562. Claims 1-7 and 9-11 were provisionally rejected on the grounds of nonstatutory obviousness-type double patenting as allegedly being unpatentable over claims 14, 15, 18, and 19 of copending Application No. 11/271,090. Claims 1-7 and 9-11 were provisionally rejected on the grounds of nonstatutory obviousness-type double patenting as allegedly being unpatentable over claims 1-7 of copending Application No. 10/499,298.

To expedite prosecution, a terminal disclaimer is enclosed herewith to overcome the provisional nonstatutory obvious type double patenting rejection, over copending applications 10/234,007; 10/790,562; 11/271,090 and 10/449,298.

Claims 1-7 and 9-11 were provisionally rejected on the grounds of nonstatutory obviousness-type double patenting as allegedly being unpatentable over claims 28-50 of copending Application No. 11/039,767.

Copending application No. 11/039,767 was filed January 18, 2005. The present application was filed earlier than copending application No. 11/039,767. The conflicting claims in application No. 11/039,767 have not in fact been patented. Applicants will address these issues in the conflicting applications if required, once the present claims have otherwise been found patentable.

Furthermore, MPEP §804 provides:

> If a "provisional" nonstatutory obviousness-type double patenting (ODP) rejection is the only rejection remaining in the earlier filed of the two pending applications, while the later-filed application is rejectable on other grounds, the examiner should withdraw that rejection and permit the earlier-filed application to issue as a patent without a terminal disclaimer.

Accordingly, in view of the amendments and further arguments provided herewith, if the provisional nonstatutory double patenting rejections are the only remaining rejections in the present application, the rejections should be withdrawn to allow the present application to issue as a patent.

Claims 1, 2, 4, 6, and 7 and 35 U.S.C. § 102

Claims 1, 2, 4, 6, and 7 were rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by Ruben et al. Applicants respectfully traverse these rejections as hereinafter set forth.

Claims 2 and 4 have been cancelled, rendering the rejection moot as to them. Claim 1 has been amended to incorporate the elements of claim 5, so that the claimed cell should be a cell derived from a PER.C6 ™ cell. This is not disclosed in Ruben et al., and hence the claim as amended is novel over Ruben et al. Indeed, the Examiner has already recognized the novelty of this subject matter, since no novelty rejection was raised for claim 5. In addition, claim 1 recites that the cell has been obtained by stable transfection of the cell with the recombinant nucleic acid encoding the IgA molecule in expressible format, implying that the recombinant nucleic acid has been integrated into the genome of the cell. Ruben et al. does not disclose this element, and also for this reason claim 1 as amended is novel over Ruben et al.

Claims 6 and 7 are not anticipated by Ruben et al. for, *inter alia*, depending from claim 1 which is not anticipated. Hence, it is submitted that the claims, also in view of the amendments, are novel over Ruben et al., and reconsideration of the claims is therefore respectfully requested.

Claims 1-7 and 35 U.S.C. § 103

Claims 1-7 were rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Ruben et al. in view of Fallaux *et al.* (WO 97/00326) (hereinafter "Fallaux et al."). Specifically, it was thought that Ruben et al. does not teach such a cell wherein said cell is derived from a cell deposited under ECACC number 96022940. It was suggested that Fallaux et al. teaches those elements. Applicants respectfully traverse these rejections as hereinafter set forth.

Claim 1 has been amended to incorporate the elements of claim 5, and further incorporate the element that the cell is obtained by stable transfection of the cell with the recombinant nucleic acid encoding the IgA molecule. This implies that the nucleic acid that encodes IgA is integrated into the genome of the cell. It is submitted that claim 1 as amended is not obvious over Ruben et al. in view of Fallaux et al., for at least the following reasons, which were explained in more detail at the personal interview.

Ruben et al. in view of Fallaux et al. does not teach or suggest all the claim limitations

The current application relates to the production of recombinant IgA by stable transfection of the nucleic acid that encodes IgA into PER.C6 cells. In contrast, Ruben et al. mentions the use of an adenovirus that contains the genetic information coding for an IgA molecule for producing IgA, wherein the adenovirus that lacks the wild-type E1-region is complemented in a 293 cell. Clearly, producing an adenovirus is something completely different from stably producing a recombinant protein such as an IgA molecule. For instance, an adenovirus will not integrate into the genome of the cells (*see e.g.*, Fallaux et al., page 5 line 10-11), whereas the currently claimed cells contain the nucleic acid encoding the IgA as part of their genome. Moreover, an entity (an adenovirus) that is in itself fully unrelated to the desired IgA is produced when the procedure of Ruben et al. is followed. The present invention is not directed to producing adenovirus, which is clear from the specification and examples, and is, for instance, also clear from the text of the incorporated application 09/549,463 (now US Patent 6,855,544) of which the present application claims continuation-in-part status. The text, for instance, in column 4 lines 22-28 (numbering as in US Patent 6,855,544), explains that adenovirus structural proteins should be absent when producing proteins according to the invention. Hence, the present invention pertains to the stable production of IgA in the absence of adenovirus production, contrary to Ruben et al.

No suggestion or motivation exists to modify or combine the referenced teachings

Furthermore, in contrast to the present application, Fallaux et al. relates to the field of gene therapy using materials derived from recombinant adenovirus. In particular, when E1-deleted adenoviruses are to be produced, they are complemented for their loss of essential E1-functions. Therefore, E1 is generally provided *in trans*, by expressing E1 from so-called packaging cells, wherein the E1-deleted adenovirus can then be propagated. The most well-known and widely used packaging cells prior to Fallaux et al. were the HEK293 cells. One particular problem with using 293 cells was that recombination between the genome of the packaging cell and the E1-deleted adenovirus vector genome was possible, because of overlap between E1 sequences present in the genome of the 293 cells and of the adenovirus vector. This

recombination led to the formation of so-called replication competent adenovirus (RCA), which has a safety related concern when E1-deleted adenovirus is to be administered to humans, *e.g.*, in gene therapy. RCA basically contains E1-sequences that were "rescued" from the genome of the complementing cells. Fallaux et al. is devoted to solving this problem, by providing a combination of a novel packaging cell and an E1-deleted adenovirus vector, which vector does not have overlap with the E1 sequences in the genome of the packaging cell. The packaging cells of Fallaux et al. are characterized in that they contain in their genome nucleic acid encoding the adenovirus E1A and E1B proteins but not pIX sequences, which enabled the formation of a packaging cell combined with a recombinant E1-deleted adenovirus vector without overlap and hence without forming RCA. The most preferred cell exemplified in Fallaux et al. for this purpose was the PER.C6™ cell. Clearly, Fallaux et al. relates to teaching a solution for preventing the problem of RCA generation when propagating recombinant E1-deleted adenovirus, and the characterizing feature of the PER.C6 ™ cells disclosed in Fallaux et al. is the presence in the genome of nucleic acid encoding E1A and E1B but not pIX protein, thereby solving that problem.

Fallaux et al. is therefore related to solving that specific adenovirus-related problem of the formation of RCA, and would be read as such by the skilled person. It is submitted that there is not a single sentence in Fallaux et al. that mentions or even remotely suggests the use of the packaging cells disclosed therein for the purpose as presently claimed, *i.e.* for the production of recombinant proteins such as IgA by stable transfection (*i.e.* allowing incorporation into the genome of the cells, and without any requirement for or even reference to adenovirus replication in the cells) of the genetic information encoding the IgA.

Hence, the Fallaux et al. reference is considered to be in a different field, and will not provide any indication to the skilled person that the cells disclosed therein, such as the PER.C6 cells, would be useful in the production of IgA by stable transfection of the nucleic acid encoding the IgA (*i.e.*, in the absence of production of adenovirus). Applicants will not dispute that one of ordinary skill in the art, based upon Fallaux et al., may have had good reasons to substitute the PER.C6™ cells of Fallaux et al. for the 293 cells of Ruben et al. for production of an adenovirus vector with E1-deletions, as asserted in the Office Action on page 26 second and third paragraphs. However, it is submitted that the Fallaux et al. reference in no way would give any

reason to substitute a 293 cell with a PER.C6™ cell outside the field of adenovirus production, as currently claimed, viz. when cells are stably transfected with a nucleic acid encoding IgA. Hence, the person skilled in the art would not in an obvious manner combine Ruben et al. and Fallaux et al. to arrive at the subject matter as currently claimed in amended claim 1.

Since no motivation exists to combine Ruben et al. with Fallaux et al., and the combined references do not teach or suggest all the claim elements of claim 1, a *prima facie* case of obviousness has not been established for claim 1. Claims 2-5 have been canceled rendering the rejections as to them moot. Claims 6 and 7 are not obvious for, *inter alia*, depending from nonobvious claim 1.

It is respectfully requested that the rejections under 35 U.S.C. § 103 be withdrawn in view of the amendments and the arguments above.

Allowable Subject Matter

It was noted by the Examiner that claim 8 contains allowable subject matter. Claim 8 has been amended to include the elements of claim 1 and thus independent.

Applicants believe that the amended claims are now allowable, and a notice of allowance is kindly solicited. If questions remain after consideration of the foregoing, the Office is kindly requested to contact applicants' agent at the address or telephone number given herein.

Respectfully submitted,

Li Feng, Ph.D.
Registration No. 57,292
Agent for Applicants
TRASKBRITT, PC
P.O. Box 2550
Salt Lake City, Utah 84110-2550
Telephone: 801-532-1922

Date: June 5, 2006

Enclosures: Budapest Treaty Declaration
Deposit Receipt
Terminal disclaimer
Information Disclosure Statement
Check in the amount of $130
Check in the amount of $180

Document in ProLaw

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Jones *et al.*

Serial No.: 10/644,256

Filed: August 20, 2003

For: EFFICIENT PRODUCTION OF IgA IN RECOMBINANT MAMMALIAN CELLS

Examiner: W. Schlapkhol, Ph.D.

Group Art Unit: 1636

Attorney Docket No.: 2578-6077US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: _____

Date of Deposit with USPS: _____

Person making Deposit: _____

BUDAPEST TREATY DECLARATION

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Ronald Brus, hereby certify that I am the Chief Executive Officer of Crucell Holland BV ("Crucell") and declare that:

1. I am informed and believe that Crucell is the assignee of U.S. patent application 10/644,256 entitled EFFICIENT PRODUCTION OF IgA IN RECOMBINANT MAMMALIAN CELLS.

2. As evidenced by the attached Deposit Receipt, I am informed and believe that Crucell's predecessor in interest made a deposit of the PER.C6™ cell line, under ECACC deposit number 96022940 under the provisions of the Budapest Treaty with the Centre for Applied Microbiology and Research Authority (European Collection of Animal Cell Cultures), Porton Down, Salisbury, Wiltshire SP4, OJG, United Kingdom, an International Depository Authority, on February 29, 1996.

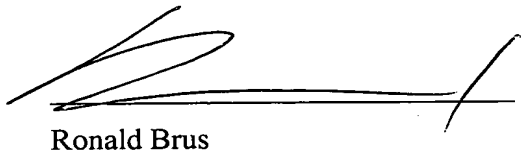
3. On behalf of Crucell, I state that all restrictions upon public access to the deposit (except those restrictions permitted by 37 C.F.R. § 1.808(b)) will be irrevocably removed upon the grant of a U.S. patent on this U.S. patent application, and the deposit will be replaced if viable samples cannot be dispensed by the depository.

4. I am informed and believe that the deposit was accepted by the International Depository Authority, effective at least as early as February 29, 1996.

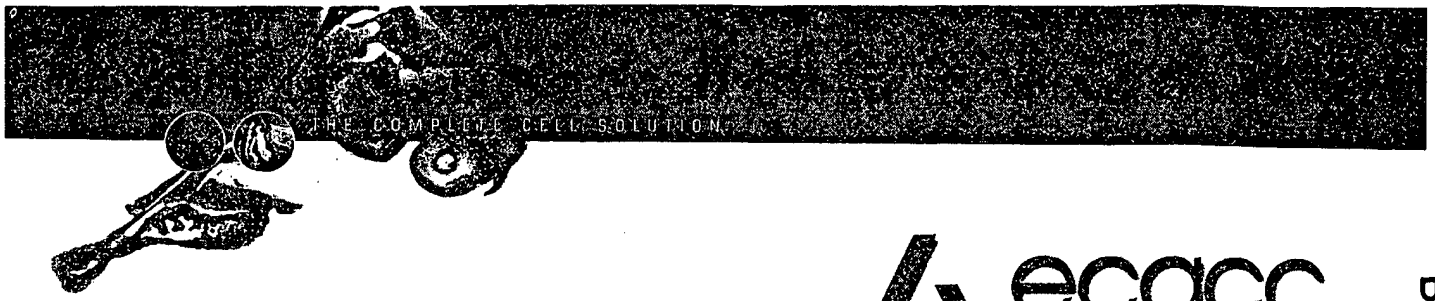
5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the U.S. Code and that such willful false statements may jeopardize the validity of the patent.

6. Attached is a copy of the deposit receipt for ECACC deposit number 96022940.

Date: 18 May 2006



Ronald Brus



ecacc
European Collection of Cell Cultures

BEST AVAILABLE COPY

Centre for Applied Microbiology and Research
&
European Collection of Cell Cultures

This document certifies that Cell Culture
(Deposit Ref. 96022940) has been accepted as a patent deposit,
in accordance with
The Budapest Treaty of 1977,
with the European Collection of Cell Cultures on 29TH February 1996

P J Packer

Dr P J Packer
Quality Manager, ECACC

CAMR
Today's Research Tomorrow's Health

European Collection of Cell Cultures, CAMR, Salisbury, Wiltshire SP4 0JG UK.
Tel: 44 (0) 1980 612512 Fax: 44 (0) 1980 611315 Email: ecacc@camr.org.uk Web Site: ecacc.org.uk

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

CRUCELL HOLLAND B.V.
ARCHIMEDESWEG 4
PO BOX 2048
2301 CA LEIDEN
THE NETHERLANDS

NAME AND ADDRESS
OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: PER C6	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: 96022940

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] A scientific description

[ ] A proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I above, which was received by it on 29th February 1996 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depository Authority on (date of the original deposit) and
A request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

IV. INTERNATIONAL DEPOSITORY AUTHORITY

Name: Dr P J Packer

Address: ECACC
CAMR
Porton Down
Salisbury SP4 OJG

Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized officials(s):

Date: 26/2/01 PJ Packer

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

CRUCELL HOLLAND B.V.
ARCHIMEDESWEG 4
PO BOX 2048
2301 CA LEIDEN
THE NETHERLANDS

VIABILITY STATEMENT
Issued pursant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY OF STATEMENT
IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: CRUCELL HOLLAND B.V. Address: ARCHIMEDESWEG 4 PO BOX 2048 2301 CA LEIDEN THE NETHERLANDS	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY: 96022940 Date of the deposit or of the transfer: 29th February 1996

II. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on [2]. On that date, the said microorganism was

[X] [3] viable

[ ] [3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2 (a) (ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Dr P J Packer ECACC CAMR Address: Porton Down Salisbury Wiltshire SP4 0JG	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 26/2/01 PSlade

4 Fill in if the information has been requested and if the results of the test were negative.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Jones et al.

Serial No.: 10/644,256

Filed: August 20, 2003

For: EFFICIENT PRODUCTION OF IgA IN RECOMBINANT MAMMALIAN CELLS

Confirmation No.: 6153

Examiner: W. Schlapkohl

Group Art Unit: 1636

Attorney Docket No.: 2578-6077US

CERTIFICATE OF MAILING

I hereby certify that this correspondence along with any attachments referred to or identified as being attached or enclosed is being deposited with the United States Postal Service as First Class Mail on the date of deposit shown below with sufficient postage and in an envelope addressed to the Mail Stop Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA22313-1450.

June 5, 2006
Date

Signature

Li Feng
Name (Type/Print)

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

06/09/2006 BARRAHA1 00000032 10644256
01 FC:1806 180.00 OP

Sir:

In compliance with the duty to disclose information material to patentability pursuant to 37 C.F.R. § 1.56, it is respectfully requested that this Supplemental Information Disclosure Statement be entered and the documents listed on attached Form PTO/SB/08 be considered by the Examiner and made of record. Copies of any cited foreign patents, publications, or pending unpublished U.S. applications are enclosed pursuant to 37 C.F.R. § 1.98(a)(2).

U.S. Patent Documents

U.S. Patent No.	Publication Date	Patentee
US - 5,631,158	05-20-1997	Dorai et al.
US - 6,475,753	11-05-2002	Ruben et al.

Other Documents

CARAVOKYRI et al., "Constitutive Episomal Expression of Polypeptide IX (pIX) in a 293-Based Cell Line Complements that Deficiency of pIX Mutant Adenovirus Type 5," Journal of Virology, November 1995, pp. 6627-6633, Vol. 69, No. 11.

YALLOP et al., "PER.C6® Cells for the Manufacture of Biopharmaceutical Proteins," Modern Biopharmaceuticals, ED. J. Knablein, 2005, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

This Supplemental Information Disclosure Statement is filed after the mailing date of the first Office Action on the merits.

The fee pursuant to 37 C.F.R. § 1.17(p) is enclosed.

Respectfully submitted,

Li Feng, Ph.D.
Registration No. 57,292
Attorney for Applicants
TRASKBRITT, P.C.
P.O. Box 2550
Salt Lake City, Utah 84110-2550
Telephone: 801-532-1922

Date: June 5, 2006
LF/bv

Enclosures: Form PTO/SB/08
Cited Non-U.S. Patent Documents
Check in the amount of $180.00

Document in ProLaw

PTO/SB/08A (10-01)
Approved for use through 10/31/2002. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	10/644,256
Filing Date	August 20, 2003
First Named Inventor	Jones et al.
Group Art Unit	1636
Examiner Name	W. Schlapkohl
Attorney Docket Number	2578-6077US

U.S. PATENT DOCUMENTS

Examiner Initials *	Cite No.[1]	Document Number Number - Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US- 5,631,158	05-20-1997	Dorai et al.	
		US- 6,475,753	11-05-2002	Ruben et al.	
		US-			
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FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] - Number[4] - Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST. 16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

Burden Hour Statement: This form is estimated to take 2.0 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

PTO/SB/08B(10-03)
Approved for use through 7/31/2006. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 2 of 2

Complete if Known	
Application Number	10/644,256
Filing Date	August 20, 2003
First Named Inventor	Jones et al.
Group Art Unit	1636
Examiner Name	W. Schlapkohl
Attorney Docket Number	2578-6077US

NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
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Constitutive Episomal Expression of Polypeptide IX (pIX) in a 293-Based Cell Line Complements the Deficiency of pIX Mutant Adenovirus Type 5

CALLIOPE CARAVOKYRI AND KEITH N. LEPPARD*

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom

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The human adenovirus type 5 capsid is composed of a number of distinct polypeptides. It has been shown previously that one of these, polypeptide IX (pIX), is not absolutely required for the production of viable virus. However, viruses lacking this polypeptide have a significantly reduced packaging limit and, in the one case studied, also show a thermolabile virion phenotype. This report describes the use of eukaryotic episomal vectors based on the Epstein-Barr virus replicon to generate cells which stably express pIX. These cells provide pIX that is efficiently incorporated into virions that are genetically pIX$^-$; such particles show enhanced thermostability. These cells have also been used to isolate a genetically pIX$^-$ virus having a genome of length some 2.3 kbp in excess of the previously defined packaging limit for pIX$^-$ virus; the resulting virions have wild-type thermostability. These cells expand the theoretical capacity of adenovirus vectors for foreign DNA to around 9.2 kbp and may therefore be useful in gene therapy applications in which vector capacity is limiting.

Over the past decade, adenoviruses (Ads) have been used increasingly as vectors for foreign protein-coding sequences. Much of this work has involved human Ad type 5 (Ad5). The Ad5 particle, which carries a double-stranded DNA genome of 35,938 bp, is formed by the association of a number of virus-encoded proteins in a precise spatial array; the resulting capsid has icosahedral symmetry with fibers protruding from each vertex (reviewed in reference 14). The faces of the icosahedron are formed principally of the hexon polypeptide. This is assembled into trimeric hexon capsomeres that further self-assemble into a group of nine hexons (reviewed in reference 24). The hexon capsomere interactions within a group of nine hexons are stabilized by other minor capsid components, including polypeptide IX (pIX). This protein has been localized, as trimers, to four crevices at hexon capsomere interfaces within a group of nine hexons (28, 29, 34).

The Ad5 genome contains a number of transcribed regions designated either early (E) or late on the basis of the timing of expression during the lytic cycle (reviewed in reference 14). pIX mRNA is transcribed from a promoter that is embedded in the E1B gene (Fig. 1A). Maximal pIX expression is normally achieved at late times postinfection, when the increased template copy number circumvents occlusion of the pIX promoter due to overlapping E1B transcription (33). An Ad5 pIX$^-$ mutant, *dl*313, which has a large deletion removing the pIX gene, as well as adjacent E1A and E1B sequences (16), can be grown to reasonable titers in 293 cells which complement its E1A and E1B deficiencies (9). Although 293 cells contain the pIX gene, it is expressed only at low levels that are not sufficient to complement pIX deficiency (4, 6, 30). As a result, *dl*313 particles grown in 293 cells lack pIX. Thus, pIX is not essential for particle formation. However, such pIX$^-$ *dl*313 particles show decreased thermostability compared with the wild type (4).

Insertion of foreign sequences into Ad5 generally requires a compensating deletion of viral DNA, since the virus particle has limited capacity for additional DNA. This maximum capacity is altered by pIX status. Thus, while pIX$^+$ virions can accommodate 1.5 to 2.0 kbp of DNA in excess of the normal genome length (in total, approximately 105% of the normal length), pIX$^-$ virions have a maximum capacity that is approximately 2 kbp less than the normal length (1, 6). To remain within this limit, viral DNA from any or all of three regions can be deleted. Replacement of up to 2.69 kbp from the E3 region, products of which are involved in modulating the host immune response (36), gives rise to replication-competent recombinants which grow to high titers in tissue culture (1, 10). Also, functional redundancy of products encoded by region *E4* allows removal of ~1.2 kbp encompassing open reading frames 1 to 4 without adverse effects on virus viability (2, 15). Finally, ~3 kbp of regions E1A and E1B can be replaced to give recombinants which are defective for growth in the absence of complementation by 293 cells.

Currently available Ad5 vectors have a capacity for a maximum of 8.5 kbp of foreign DNA. However, to ameliorate the adverse host immune responses which have been observed in animal experiments and human trials (37), it may be useful to reincorporate some E3 sequences; this gene has been shown to moderate the pathology of infection in an animal model (7). Also, certain sequences which are of potential significance for gene therapy, such as the human dystrophin cDNA (17), are too long to be accommodated within such vectors. Thus, the capacity of Ad5 vectors for exogenous DNA is one of the factors which limit their application and further development as gene therapy vectors. To begin to address this problem, we isolated a derivative of the 293 cell line which expresses high levels of Ad5 pIX and used this derivative to complement the pIX deficiency of *dl*313 virions in vivo. With this cell line, we also constructed a novel pIX$^-$ virus with a genome of greater than standard length which is defective for packaging in 293 cells.

* Corresponding author. Mailing address: Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K. Phone: (44) 1203 523579. Fax: (44) 1203 523701. Electronic mail address: LK @dna.bio.warwick.ac.uk.

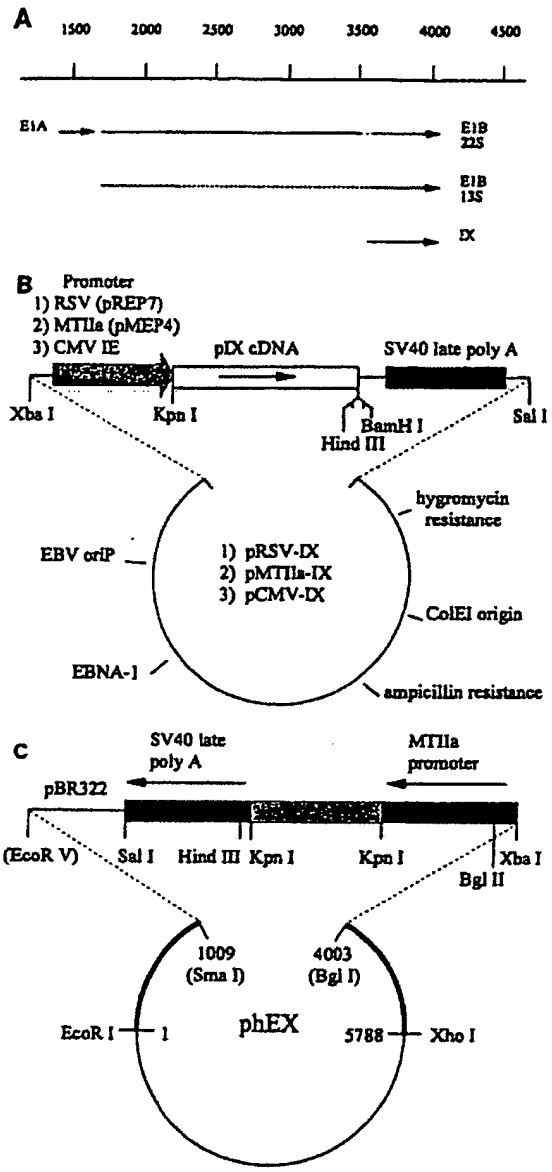

FIG. 1. (A) Transcription map of the Ad5 genome in the E1B-IX region. Nucleotide positions are numbered from the left end of the genome. Arrows indicate the principal E1B and IX mRNAs. The 3' end of mRNA from the upstream E1A gene is also shown. (B) Structures of pIX eukaryotic expression plasmids pRSV-IX, pMTIIa-IX, and pCMV-IX. These plasmids derive from pREP7 and pMEP4 (Invitrogen). The positions on the plasmids of the Epstein-Barr virus origin of replication (oriP) and EBNA-1 gene and of an expression cassette encoding resistance to hmB are indicated. Only those restriction sites that are significant to the discussion are shown. (C) Structure of the Ad5 left-end replacement plasmid, phEX. Thin lines represent pBR322, and thick lines represent Ad5 sequences. Nucleotide positions are numbered from the left end of Ad5. Stuffer sequences derived from pMEP4 are shown as filled boxes, and those derived from phage λ are represented by the hatched box. Only those restriction sites that are significant to the discussion are shown. RSV, Rous sarcoma virus; CMV; cytomegalovirus; IE, immediate early; SV40, simian virus 40.

MATERIALS AND METHODS

Cloning of pIX DNA. The pIX open reading frame was amplified by PCR with an Ad5 *Bgl*II-*Dra*I fragment (Ad5 genome positions 3328 to 4027) as the template. Primers used were 5' end-GGTACCGCCGCCGCCATGAGCACC and 3' end-CTTAAGCTTGTTTTAAACCGCATTGGG, which are homologous to Ad5 positions 3598 to 3617 and 4035 to 4017, respectively, and carry sites for restriction enzymes *Kpn*I and *Hin*dIII at their 5' ends. PCR conditions were 94°C for 2 min, 55°C for 2 min, and 74°C for 80 s for 35 cycles with 150 ng of the template and 1 U of Vent DNA polymerase (New England Biolabs) in the reaction buffer supplied. The PCR product was end filled with the Klenow fragment of DNA polymerase I, purified from agarose gels, and cloned at the *Sma*I site of Bluescribe (pBS; Stratagene), creating pBS-IX. Suitable fragments were subcloned into M13mp18/19 and sequenced with Sequenase 2.0 (U.S. Biochemicals).

The pIX sequence was excised from pBS-IX with *Kpn*I and *Hin*dIII and inserted between the same sites within the multiple cloning site regions of pREP7 and pMEP4 (Invitrogen), downstream of the Rous sarcoma virus and human metallothionein IIa (MTIIa) promoters, respectively. An analogous expression plasmid, pCMV-IX, was generated by inserting a *Cla*I-*Xho*I fragment from pCMVS5K (35), containing the human cytomegalovirus immediate-early (IE) enhancer-promoter region, in place of the *Xba*I-*Kpn*I promoter fragment in pMTIIa-IX (Fig. 1B).

Preparation of pIX-specific antiserum. pIX DNA was excised from pBS-IX with *Kpn*I and *Hin*dIII, end filled with T4 DNA polymerase, and inserted into the end-filled *Bam*HI site of glutathione *S*-transferase expression plasmid pGEX2T (Pharmacia). The predicted 39-kDa glutathione *S*-transferase–pIX fusion protein was readily detectable in the soluble fraction of lysates of *Escherichia coli* transformed with pGEX-IX, after induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 to 5 h at 37°C (data not shown). Large-scale purification of the fusion protein was performed by binding to glutathione-Sepharose (Pharmacia) for 3 to 4 h at room temperature and elution, after extensive washing, with 20 mM glutathione in 50 mM Tris HCl (pH 8.0) at 4°C overnight. The eluate was then dialyzed overnight against 50 mM Tris HCl (pH 8.0).

A polyclonal antiserum was raised against this purified protein by subcutaneous injection into an outbred half-lop rabbit. Approximately 400 μg of purified fusion protein, in Freund's complete adjuvant, was used for the initial immunization, which was followed by four booster immunizations with 200 to 300 μg of protein in Freund's incomplete adjuvant in each case. Serum was obtained about 10 days after each booster immunization and tested for pIX reactivity by immunoprecipitation and Western blotting (immunoblotting).

Cells and viruses. 293 cells (9) were maintained in Dulbecco modified Eagle medium (DMEM) plus 10% newborn calf serum. 293-EBNA cells (Invitrogen) stably express the Epstein-Barr virus EBNA-1 protein and were maintained in DMEM plus 10% newborn calf serum supplemented with 250 μg of geneticin (G418 sulfate; Gibco-BRL) per ml. C34 cells are a derivative of 293-EBNA which expresses Ad5 pIX (isolated as described below) and were maintained in DMEM plus 5% fetal calf serum (FCS) supplemented with 250 μg of geneticin per ml and 200 μg of hygromycin B (hmB; Boehringer) per ml. Virus-infected C34 cells were maintained in DMEM plus 2% FCS supplemented with 250 μg of geneticin per ml and 50 μg of hmB per ml.

Ad5 *dl*309, *dl*310, and *dl*313 have been described previously (16). Ad5 *dl*310X was generated by homologous recombination in vivo between pΔ310X/XhoI-C and *Xba*I-*Cla*I-digested *dl*309 viral DNA. pΔ310X/XhoI-C was constructed from pΔ310/XhoI-C (containing *Xho*I fragment C of Ad5 *dl*310 cloned between the *Eco*RI site of pBR322 and an *Xho*I site at approximate pBR322 position 2490) by insertion of an *Xba*I 8-mer linker at the *Bgl*I site (rendered blunt ended with T4 DNA polymerase) at Ad5 position 3996 within the C-terminal portion of the pIX coding region (18). Virus stocks were grown routinely, and titers were determined by plaque assay on 293 cells. For plaque assays on C34 cells, selective agents were omitted from the agar overlays. Purified virions were prepared from infected-cell lysates by CsCl density gradient centrifugation, and particle concentrations were determined by measuring the A_{260} of virion samples lysed in 0.1% sodium dodecyl sulfate (SDS). One A_{260} unit represented 10^{12} particle equivalents per ml of lysed sample.

Standardized virion samples were prepared for protein analysis by dilution in radioimmunoprecipitation assay buffer (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride), for DNA analysis by proteinase K digestion and phenol-chloroform extraction, and for infectivity assays by fivefold dilution in 0.1% bovine serum albumin–50% glycerol–10 mM Tris HCl (pH 8.0)–100 mM NaCl–2 mM $MgCl_2$. Transient expression assays were performed by transfecting approximately 9×10^6 293 cells with 17 μg of plasmid DNA by calcium phosphate coprecipitation. Cell extracts were prepared 48 h posttransfection. Gene expression in infected cells was routinely assayed at 24 h postinfection.

Protein and RNA analysis. When appropriate for protein analysis, cells were labelled with [^{35}S]methionine (1,000 Ci/mmol; Amersham) for 2 h prior to harvest (0.1 mCi/ml, 10^7 cells). Cells were lysed in radioimmunoprecipitation assay buffer, extracts were immunoprecipitated with the relevant antibodies, and precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) with a 15% polyacrylamide gel (175:1 acrylamide-bisacrylamide ratio).

Labelled proteins were detected by autoradiography with Fuji RX film. Western analysis was performed as previously described (3, 32). For cytoplasmic RNA analysis, cells were extracted with isotonic buffer containing Nonidet P-40 as previously described (19). pIX mRNA was detected in 50-μg samples of purified total cytoplasmic RNA by RNase protection assay (22) by using as the probe labelled, in vitro-transcribed RNA from a 665-bp *Pst*I-*Sal*I fragment of pRSV-IX cloned in pGEM3 (Promega). A 397-base fragment of this probe is protected by the 3′ portion of pIX mRNA expressed from each of the pIX expression vectors; E1B mRNA protects a 225-base fragment of the same probe.

Isolation and analysis of pIX-expressing cell lines. 293-EBNA cells (7.5×10^6) were transfected overnight by calcium phosphate coprecipitation with 17 or 34 μg of each pIX expression plasmid. Following glycerol shock, transfected monolayers were subcultured twice over a 4-day period at a low dilution in DMEM plus 10% FCS containing 250 μg of geneticin per ml. Thus, the originally transfected monolayers gave rise to several independent cell populations which were then subjected to selection with various concentrations of hmB (50 to 350 μg/ml). Culture media were replaced every 3 to 4 days until hmB-resistant colonies were apparent (14 to 35 days, depending on the cell line and hmB concentration). The resistant cells present at each hmB concentration were pooled and expanded by passaging five or six times in DMEM plus 10% FCS containing the appropriate concentration of hmB. Cell samples were tested for pIX expression at each passage by Western blotting and subsequently maintained as described earlier.

Construction of recombinant virus CES. A series of heterologous DNA fragments was assembled in pΔ310X/XhoI-C in place of E1A, E1B, and pIX sequences. First, a 910-bp *Kpn*I-*Xba*I fragment from pMEP4 (containing the MTIIa promoter) replaced the *Kpn*I-*Xba*I (Ad5 positions 2048 to 3996) fragment of pΔ310X/XhoI-C to create pΔ310X/XhoI-C(M1). Next, a 450-bp *Sal*I-*Kpn*I fragment of pMEP4 [containing the simian virus 40 poly(A)$^+$ signal] was subcloned into a pBR322 derivative carrying a *Kpn*I site at bp 2065, creating pBRK-SV, and a 915-bp *Eco*RV-*Kpn*I fragment from pBRK-SV was used to replace the *Sma*I-*Kpn*I (Ad5 positions 1008 to 2048) fragment of pΔ310X/XhoI-C(M1), creating pΔ310X/XhoI-C(M2). Finally, a 1.5-kbp *Kpn*I fragment from phage λ (positions 17053 to 18556) was inserted at the *Kpn*I site of pΔ310X/XhoI-C(M2) to create phEX (Fig. 1C). A 10-μg sample of the 4.3-kbp *Eco*RI-*Xba*I fragment from phEX and 2.5 μg of *Xba*I-digested and dephosphorylated *dl*310X virus DNA were ligated overnight at 15°C with 25 U of T4 DNA ligase (Boehringer). The ligated DNA was purified and transfected into 2×10^6 C34 cells. The cells were then maintained in DMEM plus 2% FCS containing 50 μg of hmB per ml for 16 h following glycerol shock and subsequently maintained under an agar overlay. After 7 to 8 days, individual plaques were picked and amplified in C34 cells.

All genetic manipulations were assessed for risk according to the guidelines of the United Kingdom Advisory Committee on Genetic Modification and carried out under appropriate containment conditions.

RESULTS

Production of pIX cell lines. To express pIX from heterologous promoters, the pIX open reading frame was amplified from the relevant region of the Ad5 genome by PCR. The PCR product was sequenced and cloned downstream of the human cytomegalovirus I-E promoter, the Rous sarcoma virus promoter, or the inducible human MTIIa promoter in plasmids also carrying an Epstein-Barr virus-based replicon, which permits episomal maintenance in eukaryotic cells (Fig. 1B). Expression of pIX from these constructs was assessed in transient assays in 293 cells. Very high levels of both pIX mRNA (Fig. 2A, lane 2) and protein (Fig. 2B, lane 8) were produced from the cytomegalovirus construct, probably because of the transactivation of this promoter by the Ad E1a 13S mRNA translation product present in 293 cells (8). Very low pIX levels were produced by the MTIIa construct (Fig. 2A, lane 3; Fig. 2B, lane 9), reflecting the basal activity of this promoter. pIX expression from the Rous sarcoma virus construct was intermediate between these two extremes (Fig. 2A, lane 4; Fig. 2B, lane 7). The levels of endogenous 293 cell E1B mRNA and 55K protein served as controls for the number of cell equivalents assayed in each case. pIX protein levels produced by the cytomegalovirus construct were similar to those observed in extracts of *dl*309-infected cells during the late phase of infection (Fig. 2B, lanes 1 and 8), suggesting that cells stably expressing the pCMV-IX construct might provide nearly wild-type levels of pIX for virus packaging.

The pIX expression plasmids were next used to establish

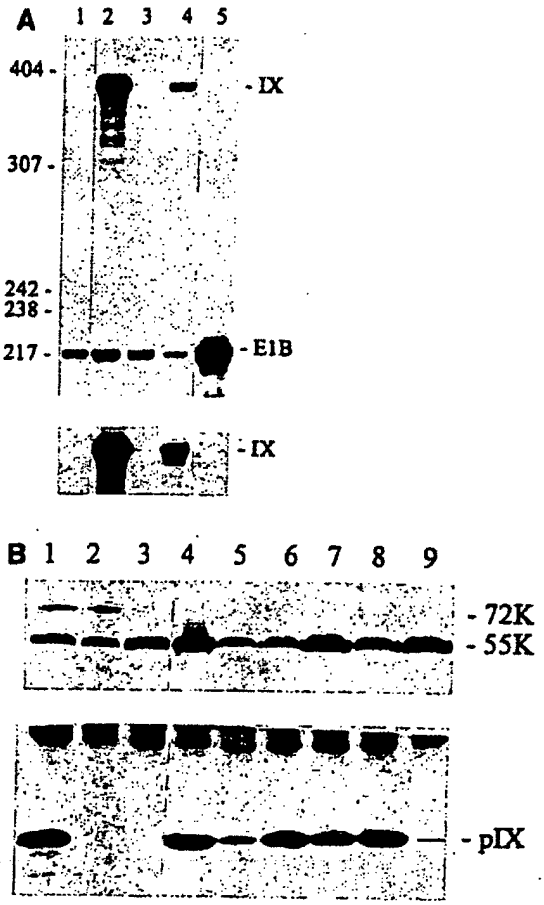

FIG. 2. (A) pIX mRNA expression from plasmids in transient assays in 293 cells, detected by RNase protection. Probe fragments were analyzed on a 5% polyacrylamide–7 M urea gel. Lanes: 1, mock-transfected cells; 2, pCMV-IX; 3, pMTIIa-IX; 4, pRSV-IX; 5, *dl*309-infected HeLa cells. The lower segment is an overexposure of the pIX-specific bands from the same experiment. The numbers on the left are molecular sizes in nucleotides. (B) pIX protein expression from plasmids in transient assays in 293 cells, and in cells derived from similar transfections of 293-EBNA cells after hmB selection, detected by Western blotting. The low-molecular-weight region of the filter was probed for pIX with anti-pIX polyclonal serum; the upper portion was similarly probed with a mixture of the E1B 55K and E2A 72K specific monoclonal antibodies (25, 27). Lanes: 1 to 3, control 293 cell infections with *dl*309 or *dl*313 or mock infection, respectively; 4 to 6, cell lines C34 (pCMV-IX) and M34 (pMTIIa-IX) and a representative line carrying pRSV-IX, respectively; 7 to 9, transient assays with pRSV-IX, pCMV-IX, and pMTIIa-IX, respectively.

cells containing a range of steady-state levels of pIX. Since preliminary experiments suggested that the EBNA-1 gene on the pIX vectors was not sufficiently active to sustain plasmid replication, a 293 cell derivative (293-EBNA) that stably expresses the EBNA-1 gene was used. Some adverse effect of pIX expression on cell viability was observed during the initial selection, with hmB, of cells containing the pIX plasmids; cell viability at high concentrations of the selective agent was inversely related to promoter strength. Cells transfected with the low-level expression MTIIa construct and those transfected with a nonexpressing control plasmid had similar growth pat-

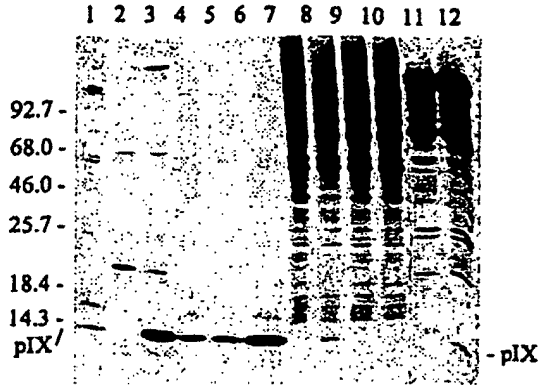

FIG. 3. Synthesis of pIX in established cell lines. [^{35}S]methionine-labelled cell extracts were analyzed by SDS-PAGE either directly (lanes 7 to 12) or following immunoprecipitation with anti-pIX serum (lanes 1 to 6). Lanes: 3 and 12, *dl*309-infected 293 cells, 24 h postinfection; 2 and 11, *dl*313; 1 and 10, M34 cells; 4 and 9, representative pRSV-IX-containing line; 5 and 7, C34/50 cells; 6 and 8, C34 cells. The positions of marker proteins are indicated at the left (molecular sizes are in kilodaltons).

terns within an hmB concentration range of 50 to 350 μg/ml, whereas cells containing pCMV-IX were adversely affected by relatively small increases in the hmB concentration and could not be isolated at concentrations above 200 μg of hmB per ml. Consequently, all three lines were maintained subsequently at a uniform concentration of 200 μg of hmB per ml unless indicated otherwise.

Cells harboring each of the three plasmids contained readily detectable pIX (Fig. 2B, lanes 4 to 6; Fig. 3, lanes 1, 4, and 6). Cells established with pCMV-IX (C34 cells) expressed levels of pIX that were detectable without immunoprecipitation (Fig. 3, lane 8) and which were similar to those found during the late phase of a wild-type virus infection (lane 12). pIX levels in C34 cells and in cells established with pRSV-IX were similar to those detected in the corresponding transient assays (Fig. 2B, lanes 4 and 8 and lanes 6 and 7), whereas pIX levels in MTIIa-IX cells (M34) were higher than those achieved by transient expression (lanes 5 and 9), suggesting that the low basal level of expression from this promoter permits the selection of a higher plasmid copy number. This was confirmed by Southern blot analysis of plasmid DNAs from the Hirt supernatants of the three cell lines. To exclude the possibility that residual excess plasmid DNA from the transfection was still present in the cultures, DNA was cut with *Dpn*I; only DNA that has been replicated after transfection is resistant to cleavage by this enzyme (11, 23). C34 cells contained only barely detectable levels of such plasmid DNA (Fig. 4, lane 6), whereas M34 cells contained plasmid levels (lane 12) that were similar to those found in a control cell line containing the vector only (lane 3); pRSV-IX-containing cells showed intermediate levels (lane 10). These data also underline the relative sensitivity of cell lines to changes in the hmB concentration, as described earlier. C34 cells passaged at a reduced hmB concentration (50 μg/ml; C34/50) showed reduced replicating plasmid levels (Fig. 4, lane 8) and reduced levels of pIX expression (Fig. 3, lane 5). Since C34 cells maintained at 200 μg of hmB per ml expressed pIX at levels equivalent to those seen late in an Ad5 infection, these cells were chosen for further analysis.

Complementation of pIX$^-$ virus. The Ad5 pIX$^-$ mutant *dl*313 was used to investigate the degree of complementation afforded by C34 and M34 cells. The presence of pIX in purified *dl*313 virions grown under either complementing or noncomplementing conditions was assessed by Western blotting (Fig. 5A). *dl*313 virions produced in C34 cells (lane 6) contained levels of pIX comparable to those found in wild-type particles (lane 4), indicating that pIX present in these cells is competent for virus packaging. This conclusion was further supported by the presence of pIX in *dl*313 virions from M34 cells (lane 7). The same virion samples were also tested for stability at 48°C in view of the fact that *dl*313 virions assembled without pIX in 293 cells rapidly disintegrate at this temperature (4). The heat inactivation profiles shown in Fig. 5B confirmed these earlier results and also indicated that *dl*313 virions containing pIX from C34 cells had significantly greater heat stability than those produced in 293-EBNA cells. This difference was, however, gradually reduced with increasing lengths of incubation at 48°C, suggesting that the presence of pIX in *dl*313 virions is not sufficient, per se, to complement fully their heat-labile phenotype. Nonetheless, it is clear that episomally produced pIX in C34 cells can be competently recruited for virion assembly during virus infection.

Construction of a novel pIX$^-$ virus. Previous studies have shown that the maximum genome length that can be packaged into Ad particles in the absence of pIX is equivalent to only 93% of the normal genome length (6). To investigate further the efficiency of incorporation of C34-produced pIX into virions, a plasmid was constructed (phEX; Fig. 1C) in which most of the coding regions for E1A and pIX, together with the whole of E1B, were replaced by non-Ad sequences. This plasmid, if recombined into an Ad5 genome, would give rise to a pIX$^-$ genome that would be larger than the wild-type length by ~330 bp, equivalent to 101% of the normal length. phEX, which retains the leftmost 1,009 bp of the Ad5 genome, including the packaging signal (12, 13), was constructed from the cloned left end region of an Ad5 variant, *dl*310X. This virus contains a single *Xba*I site near the 3' terminus of the pIX coding region (see Materials and Methods) which is predicted to alter the last 10 amino acids of pIX and to add an additional four residues (Fig. 6A). This change results in an electrophoretically variant form of pIX (Fig. 6B, lane 2). *dl*310X is otherwise similar to wild-type *dl*309 with respect to replication

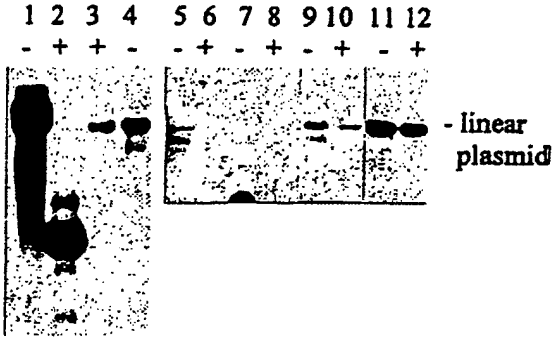

FIG. 4. Plasmid DNA, purified by Hirt extraction from established cell lines, was detected by Southern analysis with nick-translated plasmid pIX as a probe following digestion with *Hin*dIII either with (+) or without (−) *Dpn*I. Lanes: 1 and 2, control bacterially grown plasmid pREP7; 3 and 4, control cell line containing pREP7; 5 and 6, C34 cells; 7 and 8, C34/50 cells; 9 and 10, a representative pRSV-IX-containing cell line; 11 and 12, M34 cells. Lanes 1 to 4 and 5 to 12 are taken from short and long exposures, respectively, of the same blot.

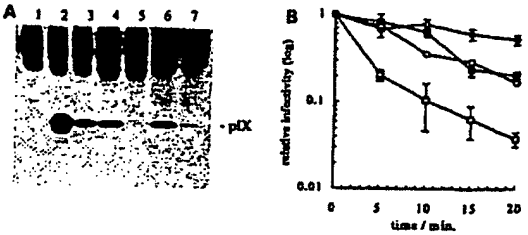

FIG. 5. pIX content and thermostability of Ad particles. (A) Lysates of $\sim 10^{11}$ purified virions were immunoprecipitated with anti-pIX serum, and pIX was subsequently detected by Western blotting following SDS-PAGE, with the same polyclonal antiserum. Lanes: 1 to 3, control 293-EBNA, C34, and M34 cells ($\sim 9 \times 10^6$ per lane), respectively; 4, *dl*309 grown in 293-EBNA cells; 5 to 7, *dl*313 grown in 293-EBNA, C34, and M34 cells, respectively. (B) Samples of the purified Ad particles assayed for pIX in panel A were diluted in Tris-buffered saline–2% calf serum and incubated at 48°C for various periods of time. Residual infectivity was determined by plaque assay on 293-EBNA cells and is expressed as a proportion of initial infectivity, plotted on a logarithmic scale. The values shown are the averages of three separate determinations. Standard deviations are shown in three cases; error bars for M34-grown *dl*313 were omitted for clarity. The stability of M34-grown *dl*313 was not significantly different from that of C34-grown *dl*313. Symbols: ○, *dl*309 grown in 293-EBNA cells; □, *dl*313 grown in 293-EBNA cells; ■, *dl*313 grown in C34 cells; ○, *dl*313 grown in M34 cells.

and virus production (31). Ligation of the *Eco*RI-*Xba*I fragment from phEX to *dl*310X viral DNA cleaved at the single *Xba*I site, followed by DNA transfection into C34 cells, produced the variant virus CE5.

Restriction analysis of DNA extracted from purified CE5 virions grown in C34 cells showed that the encapsidated genome had the expected structure (Fig. 7A). The additional bands indicated in lanes 1 and 6 are characteristic of the phEX sequence incorporated into CE5. Other differences between CE5 and its immediate parent, *dl*310X (lanes 2, 5), namely, loss of the 2.8-kbp *Hin*dIII-G, 2.05-kbp *Kpn*I-H, and position 2803 to 3996 *Hin*dIII-*Xba*I fragments, were also as expected. The 1.0-kbp *Hin*dIII I fragment seen in lanes 2 and 3, which constitutes the right end of the genome and which was therefore expected to be present also in CE5, was visible in the original negative. The integrity of the inserted non-Ad sequences was also confirmed by Southern blotting (data not shown). Therefore, the isolation procedure has not selected for a deleted variant of the expected CE5 genome. Western blot analysis of virion proteins (Fig. 7B) confirmed the presence of pIX in CE5 virions (lane 2) at levels equivalent to those found in *dl*313 particles grown in C34 cells (lane 1). Interestingly, control *dl*310X virions grown and analyzed in parallel (lane 3) contained the wild-type pIX produced in C34 cells, as well as the variant *dl*310X pIX. Thus, the pIX in C34 cells not only assembles into virions of pIX$^-$ mutants CE5 and *dl*313 but can also be incorporated effectively in parallel with pIX expressed simultaneously from a viral genome.

The variant CE5 was isolated and propagated in C34 cells. When the titers of CE5 stocks were determined in parallel on both C34 and 293 cells, the virus showed approximately 10^5-fold reduced plaque-forming efficiency in 293 cells. However, CE5 DNA replication was comparable in both cell types (data not shown). Therefore, it appears that packaging of the CE5 genome is severely restricted by the low levels of pIX in 293 cells. We also examined CE5 particles to determine whether or not they showed normal thermostability. As shown in Fig. 7C, CE5 particles showed a stability profile similar to that of the parent, *dl*310X, while as in Fig. 5B, C34-grown *dl*313 particles were less stable than the wild type; Fig. 7D shows that in a separate experiment, *dl*310X particles grown in 293 cells were as stable as wild-type *dl*309. The difference in stability between *dl*310X and CE5 grown in C34 cells in Fig. 7C may reflect the effect on 310X of packaging in the presence of mixed wild-type pIX and *dl*310X pIX. Although variations in shape between particular heat inactivation profiles were seen in different experiments, the relative stability as presented here was consistently observed in replicate experiments. Thus, C34 cells, by providing pIX, are able to complement the lethal pIX$^-$ defect in CE5 and impart wild-type stability to the resulting particles.

DISCUSSION

In this study, we investigated the possibility that constitutive expression of pIX in a cell line would provide a functional pool of molecules for encapsidation of pIX$^-$ Ad5 genomes. The availability of plasmid vectors which contain the Epstein-Barr virus origin of episomal replication, which can thus be stably maintained in dividing, EBNA-1-containing cells (21, 26), enabled us to assess the levels of pIX required for complementation of the pIX$^-$ phenotype. Although pIX produced from all three types of episomal vector constructed was efficiently incorporated into the virions of pIX$^-$ mutant *dl*313 virions (Fig. 5A and data not shown), only C34 cells harboring the pCMV-IX plasmid provided pIX at levels comparable to those seen in wild-type virions. This finding is consistent with the fact that pIX is required stoichiometrically for stabilization of the group-of-nine hexon assemblies that make up the 20 faces of the Ad particle (5, 34). It was surprising that the C34-produced *dl*313 virions were not as stable as wild-type virions during prolonged incubation at 48°C, given their nearly wild-type levels of pIX and also the observation that CE5 particles carrying similar levels of pIX from C34 cells showed normal stability. However, other factors have been shown previously to affect virion stability. In their earlier study of *dl*313, Colby and Shenk (4) also analyzed *dl*312, a pIX$^+$ mutant with an ~0.9-kbp deletion within the E1A gene, and showed that it had a heat inactivation profile intermediate between those of the wild type and *dl*313, similar to that shown here by the pIX$^+$ *dl*313 particles produced in C34 cells. Also, Liu et al. (20) showed that a mutation in the capsid protein precursor, pVIII, resulted in reduced heat stability of virions, although the possibility that second-site mutations in structural proteins other than pIX might also contribute to *dl*313 particle instability was largely

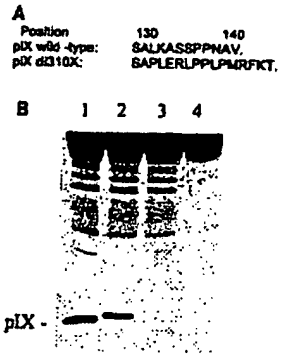

FIG. 6. (A) Comparison of the predicted structure of the C terminus of *dl*310X pIX with that of the wild-type virus. Amino acid residues are represented by the single-letter code. (B) pIX in lysates of virus-infected 293 cells, detected by Western blotting. Details are the same as for Fig. 2B. Lanes: 1, *dl*309; 2, *dl*310X; 3, *dl*313; 4, mock infection.

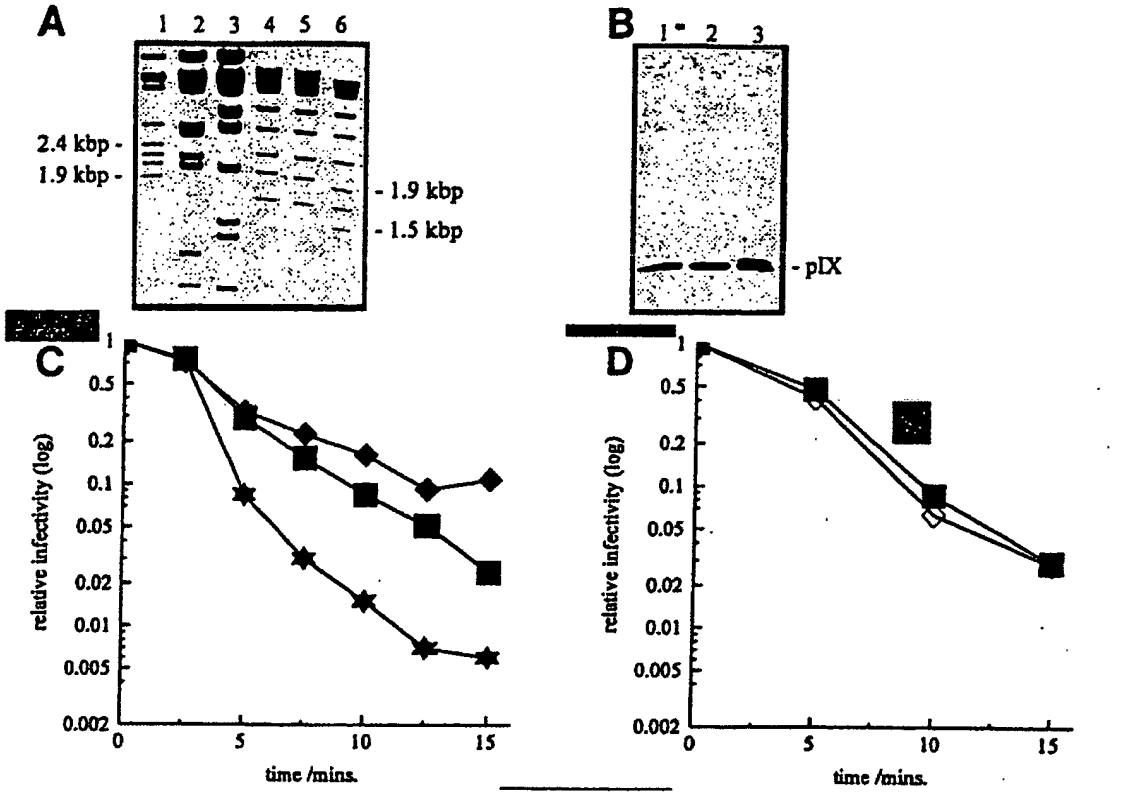

FIG. 7. (A) Restriction analysis of viral DNA from CsCl gradient-purified virions. Fragments were separated by electrophoresis in an agarose gel in 1× Tris-borate-EDTA buffer and visualized by ethidium bromide staining. Lanes: 1 to 3, *Xba*I plus *Hin*dIII; 4 to 6, *Kpn*I; 1 and 6, CE5; 2 and 5, *dl*310X; 3 and 4, *dl*309. The positions of fragments diagnostic for the correct structure of CE5 are indicated. (B) pIX immunoprecipitated from solubilized virus particles grown in C34 cells, detected by Western blotting. Details are the same as for Fig. 5A. Lanes: 1, *dl*313; 2, CE5; 3, *dl*310X. (C) Thermostability of Ad particles. Samples of the purified Ad particles analyzed for pIX in panel B were assayed as described in the legend to Fig. 5B. Each curve represents a single experiment. Symbols: ■, *dl*310X; ✱, *dl*313; ◆, CE5. (D) Same as panel C, except that particles were grown in 293 cells. Symbols: ◇, *dl*309; ■, *dl*310X.

excluded by Colby and Shenk (4). Together, these data suggest that incorporation into *dl*313 particles of wild-type levels of pIX is not the sole determinant of virion stability in the heat inactivation assay. The additional factor(s) determining particle stability may be related either to E1A status (4) or to *cis* effects of genome alterations on structural protein synthesis, particle assembly, or genome encapsidation.

Virus CE5 is a novel pIX$^-$ Ad5 variant, where the pIX coding region has been replaced by foreign DNA sequences, so maintaining a genome of greater than standard length. This alteration has no adverse effects on viability or genome integrity so long as CE5 is maintained in complementing C34 cells. In 293 cells, CE5 shows dramatically reduced plaque-forming efficiency. These results suggest that C34 cells will allow the isolation of further novel Ad5 recombinants that would not be viable in previously available cell types. Such viruses would have E1 deletions extending through the pIX region but would nevertheless be able to accommodate additional heterologous sequences up to the limit defined for wild-type Ad5 (1). In combination with deletion of nonessential E3 and E4 sequences mentioned earlier, this approach should permit the isolation of recombinants containing a total of 9.2 kbp of foreign DNA. Ultimately, the ability of C34 cells to sustain variants that are defective in packaging in other cell types may be useful in the development of packaging systems for highly defective, helper-dependent Ad5 recombinants that need contain only the minimal *cis*-acting sequences required for replication and packaging. Such recombinants could be useful in gene therapy protocols for the delivery of DNA sequences that exceed the size limits of existing Ad vector systems.

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3
PER.C6® Cells for the Manufacture of Biopharmaceutical Proteins

Chris Yallop, John Crowley, Johanne Cote, Kirsten Hegmans-Brouwer, Fija Lagerwerf, Rodney Gagne, Jose Coco Martin, Nico Oosterhuis, Dirk-Jan Opstelten, and Abraham Bout

Abstract

The PER.C6® human cell line was generated by immortalizing retina cells with the E1 genes of human adenovirus type 5. Master and Working cell banks were laid down and characterized in detail. Initially, the cell-line was used for the efficient and safe manufacture of recombinant adenoviral vectors for use in gene therapy and as vaccines. In total, six adenoviral vectors manufactured on PER.C6 are currently in clinical trials in the US and in Europe, of which one is used as a vaccine. In addition, PER.C6 is used for the manufacture of classic vaccines such as the influenza virus and West-Nile virus vaccines. The latest application of PER.C6 is in the field of protein production. A monoclonal antibody manufacture process has been developed to determine the growth and metabolic properties of PER.C6 and to investigate the yield and quality of the produced proteins. This chapter details the history of the PER.C6 cell line, the generation of antibody-producing PER.C6 cells, and the performance of these cells in production processes. In general, PER.C6 can be easily adapted to serum-free medium and can grow to very high cell concentrations in fed-batch ($>10^7$ cells mL^{-1}) and, in particular, continuous perfusion ($>10^8$ cells mL^{-1}). Specific productivity can be maintained at these high cell concentrations, resulting in high product yields. In addition, the high cell densities have no impact on product quality. Such cell densities are novel in the industry and will have a significant impact on the cost of manufacturing biopharmaceutical proteins, in particular those that are difficult to manufacture.

Abbreviations

APAC	analytical protein A chromatography
CBER	Center for Biologics Evaluation and Research
CHO	Chinese hamster ovary
cIEF	capillary isoelectric focusing
CMV	cytomegalovirus
CSPR	cell specific perfusion rate
DCW	dry cell weights
DHFR	dehydrofolate reductase
DMEM	Dulbecco's modified eagle medium
E1	transcription unit
ELISA	enzyme linked immuno sorbent assay
FBS	fetal bovine serum
G418	geneticin
HER	human embryonic retina

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HPAEC-PAD	high performance anion exchange chromatography with pulsed amperometric detection
HPLC	high-performance liquid chromatography
HP-SEC	high-performance size exclusion chromatography
IEF	Isoelectric focusing
LC	liquid chromatography
LC-MS	liquid chromatography coupled mass spectroscopy
MALDI	matrix assisted laser desorption ionization
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
MCB	Master Cell Bank
MDM2	morse double mint 2
MS	mass spectrometry
MW	molecular weight
N/D	not detected
NS0	hybrid cells
PGK	phosphoglycerate kinase
PNG	peptide:N-glycosidase
PrPsc	prior specific protein (scrapy)
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SP2	hybrid cells
UVA 280	ultaviolet absorption at a wavelength of 280 nm
VCD	viable cell density
VPR	volumetric production rate

3.1 Introduction

The PER.C6® human cell line was generated by the immortalization of primary retina cells with E1 sequences of human adenovirus serotype 5. The cell line was initially developed for the safe production of pharmaceutical grade recombinant human adenoviral vectors. Such vectors are currently used for vaccine and gene therapy purposes. In addition, the cell line is exploited for the manufacture of classical vaccines including influenza and West Nile Virus vaccines.

More recently, PER.C6 cells were evaluated for the production of therapeutic proteins, the global market of which has grown rapidly over the past five years, with an average annual growth rate of approximately 21% and sales reaching approximately $41 bn in 2003 (AS Insights 2003; Reuters Business Insight 2003). Furthermore, with approximately one-third of all pipeline candidates currently in clinical development, this growth looks set to continue. One group of therapeutic proteins – the monoclonal antibodies – has shown particularly rapid growth in recent years, increasing from approximately 1% of therapeutic protein sales in 1995 to 14% in 2001. There are currently 15 approved antibodies on the market and many more in the late stages of clinical development.

The production of therapeutic proteins is commonly performed using mammalian cell lines, most commonly Chinese hamster ovary (CHO), but including also NS0 and SP2/0 cell lines. Mammalian cell lines are currently responsible for more than 60% of all licensed products. Their importance is due to an ability to perform the correct complex post-translational modifications required by many therapeutic proteins for their physiological activity. However, a drawback of mammalian cells is that yields are typically low compared to bacterial and yeast systems, while development and manufacturing costs are high. A major goal of process development groups over recent years has therefore been the increase of product yields (from both upstream and downstream process improvements) and the reduction of development

and manufacturing costs and timelines (see also Part IV, Chapter 1).

Due to the high doses required for many antibody therapies, high product yields are particularly desirable. Yields of 1–2 g L^{-1} are current industry standard targets. Yields above 2 g L^{-1} have also been achieved, but at present these do not generally result in significant cost savings due to current limitations in downstream processing. However, as progress is made in this area, demands for higher yields can be expected. At the same time, there is also a drive to reduce development costs and timelines. One goal is to reduce the time to clinic by reducing the time taken to generate production cell lines and to generate the material for pre- and early clinical phase studies. Current timelines may vary slightly depending on the individual situation, cell line, antibody, etc., but typically range from 14 to 16 months for cDNA to production of clinical trial material. However, these may be expected to decrease in the coming years. The more efficient use of process development resources is another major driver, particularly for projects up to early clinical phase studies where the risk of failure is highest and where it may be necessary to run a number of projects in parallel.

An approach adopted by many has been the development of platform technologies. The aims of such a platform include for example, the provision of technology required to generate cell lines with high cell-specific productivity, to ensure the selection of production cell lines that perform well in the desired production process, to develop cost-effective production media, and to provide high-yielding, efficient and cost-effective production and purification processes suitable for large-scale manufacture.

Of particular importance is the development of generic processes. By developing processes that are generic, timelines can be shortened and development costs reduced for each new cell line that is generated. For example, the development of generic production and purification processes removes the need to perform lengthy and costly process development for each new cell line, thus reducing costs, timelines and allowing multiple projects to proceed simultaneously. They may also act as a basis for development of the final manufacturing process, thus minimizing investment in process development for Phase III and beyond. Moreover, the inclusion of a generic production process in the cell line generation program allows the selection of cell lines that perform optimally in the desired final production process.

It is the aim of Crucell and DSM Biologics to establish the human PER.C6 cell line as a platform for the production of therapeutic proteins, with particular emphasis on monoclonal antibodies. The approach taken has been to develop an integrated production platform that combines the rapid generation of high-yielding production cell lines with high-yielding generic production (batch, fed-batch and perfusion) and purification processes and a metabolically characterized host cell line. Data generated from the metabolic characterization of PER.C6 cell lines was used to design generic, high-yielding batch, fed-batch and perfusion production processes, matched to the metabolic requirements of the cells. Cell lines are evaluated as early as possible in the desired production process so that lead clones are selected that match and will perform optimally in the desired production process.

The investigations described in this chapter provide an overview of clone generation, fed-batch and perfusion process development, as well as detailing the history of the PER.C6 cell line, and how it has been characterized. These studies have

been conducted in an alliance between Crucell Holland N.V. (Leiden, The Netherlands) and DSM Biologics (Groningen, The Netherlands and Montreal, Canada).

3.2 Generation of PER.C6 Cells

3.2.1 Immortalization of Cells by E1 Proteins of Human Adenovirus

Human adenoviruses are associated only with mild disease in healthy humans [1]. Adenoviruses have a DNA genome of approximately 36 kb that encodes proteins of the virus capsid, and proteins that dedicate the cell to replicate the viral genome and synthesize viral proteins. Among the latter are the so-called E1 gene products of adenovirus. It has long been known that the isolated E1 genes can immortalize primary human cells [2]. This property of E1 genes of adenovirus was used to generate the PER.C6 cell line. The E1 region of adenovirus 5 consists of two transcriptional units, E1A and E1B. The E1A transcription unit encodes two proteins, which are generated by alternative splicing. The proteins

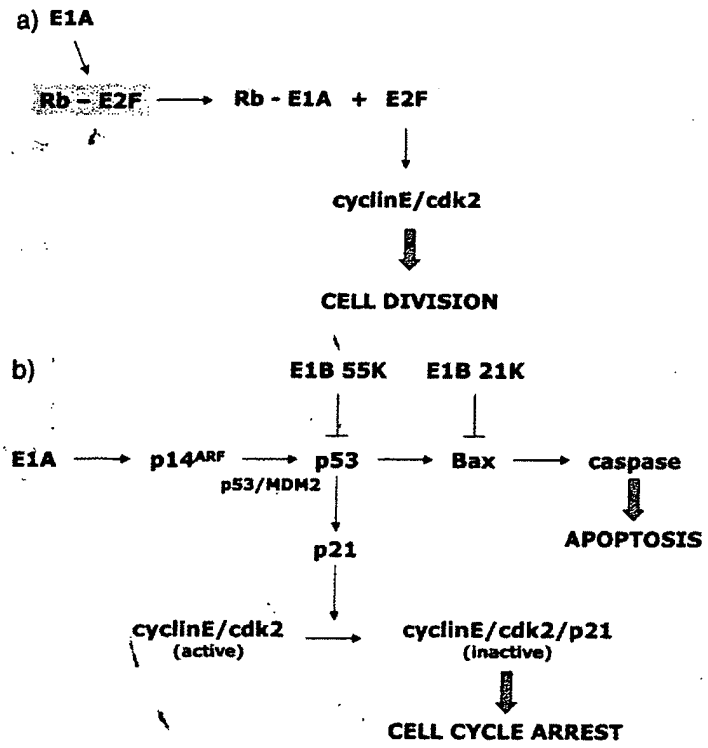

Fig. 3.1. (A) E1A-induced cell proliferation. E1A proteins release E2F from Rb, which subsequently induces CyclinE/cdk2 gene expression and pushes cells into S-phase. (B) E1A-induced cell cycle arrest and apoptosis counteracted by E1B. E1A proteins activate p53, which leads to cell cycle arrest and apoptosis. These effects are counteracted by E1B 55K, which binds directly to p53, and E1B 21K, which inactivates cytochrome-c.

are acidic in nature, are 243 and 289 amino acids long, respectively, and are located in the nucleus of the cell. A detailed description of the E1 regions of adenovirus and the function of E1A proteins of adenovirus type 5 is available on (http://www.geocities.com/jmymryk.geo/).

The E1B region generates one RNA, which is translated into two proteins, with molecular weights of 21 and 55 kDa.

For efficient immortalization and transformation of primary cells, both the E1A and E1B regions are required, although it has been described that the E1A region by itself can immortalize rodent cells [3] and occasionally human cells [4], with very low efficiency. Expression of E1A alone usually results in the induction of programmed cell death (apoptosis), which can be prevented by co-expression of E1B [5].

E1A proteins affect major cellular processes such as cell cycle control, differentiation, apoptosis, and transformation. The immortalization of primary cells occurs by binding of E1A proteins to the tumor suppressor protein pRB, p107 and p130, as well as to the co-activator p300 [6]. These proteins have in common that they can form complexes with E2F transcription factor proteins, leading to inactivation of the E2F factors. The binding of E1A leads to the release of E2F transcription factors from the complexes, which results in activation of cellular genes that have E2F binding sites in their promoters (Fig. 3.1A). Amongst these genes is cyclinE/cdk2 that stimulates the cell to enter the cell cycle. However, the strong proliferation signal of E1A causes the cell to activate p53 (Fig. 3.1B). p53 is complexed to MDM2, which renders it inactive. E1A mediates the induction of p14ARF protein expression, which inhibits the activity of MDM2, thereby causing release of p53 [7]. P53 is an activator or transcription of genes that cause cell cycle arrest and apoptosis of the cells. So E1A stimulates cells to proliferate but also induces a stress response in the cell, leading to growth arrest or apoptosis. The stress response is counteracted by the E1B proteins (Fig. 3.1B). E1B 55K, which is located in the nucleus, forms a complex with p53 and thereby inactivates it. E1B 21K interferes with the apoptotic effects of p53-induced Bax protein in the cell [8]. Bax is a pro-apoptotic protein, that causes release of cytochrome-c from mitochondria, which in turn causes caspase-mediated apoptosis. E1B 21K is a homologue of the cellular anti-apoptotic protein Bcl2 [8]. It inhibits Bax-induced release of cytochrome-c from mitochondria, thereby preventing apoptosis.

3.2.2 Generation of PER.C6 Cells

The DNA construct pIG.E1A.E1B (Fig. 3.2) was used for making PER.C6 cells. The E1 genes are driven by the human phosphoglycerate kinase (PGK) promoter, which is a known house-keeping promoter [9] and the poly(A) sequences are derived from the Hepatitis B surface antigen gene [10, 11].

The primary cells selected for transfection with the E1 construct were human embryonic retina (HER) cells, which can be immortalized relatively easily by E1 of human Ad5 [4, 12, 13] and Ad12 [14].

Fig. 3.2 DNA construct used to generate PER.C6 cells. In this construct, the E1A gene is driven by the human phosphoglycerate kinase (PGK) promoter. Transcription is terminated by the hepatitis B virus surface antigen poly(A) sequences.

Primary HER cells have a limited life span, and can be cultured only a few passages, after which the cells senesce. Transfection of HER cells with E1 constructs results in transformation and immortalization of the cells, reflected by focus formation in the cultures. This is easily recognized by both macroscopic and microscopic examination of the cultures. Such foci can be isolated and cultured further. In this way, PER.C6 cells were isolated after transfection of primary HER cells with pIG.E1A.E1B [15, 16]. The cells were apparently immortalized also, without passing through a crisis phase.

Transformation and immortalization of primary cells with E1 sequences of adenovirus guarantees: 1) a stable expression of E1 proteins, as the cells need E1 expression for growth; and 2) that no external selection marker is needed to distinguish E1 expressing from non-expressing cells.

PER.C6 cells stably express the E1 proteins. In particular, the 21K and 55K E1 proteins that counteract apoptosis and p53-mediated cell cycle arrest, respectively are expressed to high levels as compared to, for example, HEK293 cells [15]. We assume that this makes the cells relatively insensitive to apoptosis, and may be one of the factors that make the PER.C6 cells grow to high cell densities and support production of a wide variety of proteins, without further manipulation of the cells.

At passage number 29, a research Master Cell Bank was laid down, which was extensively characterized and tested for safety (including sterility testings). Research cell banks were made at passage numbers 33 and 36.

The characterization and safety testing of the cell banks has been described extensively elsewhere [16]. In brief, the identity, sterility, viral safety, absence of PrPsc protein, tumorigenicity and genetic characterization, including chromosome analysis, has been performed.

A description of the history of the cell line – as well as study protocols and reports of all safety studies carried with the cell line – has been filed as a Biologics Master File at CBER.

3.3 PER.C6 Cells for the Manufacture of Recombinant Proteins

The first step in the manufacturing train is the generation and selection of a high-producing cell line. This has been performed

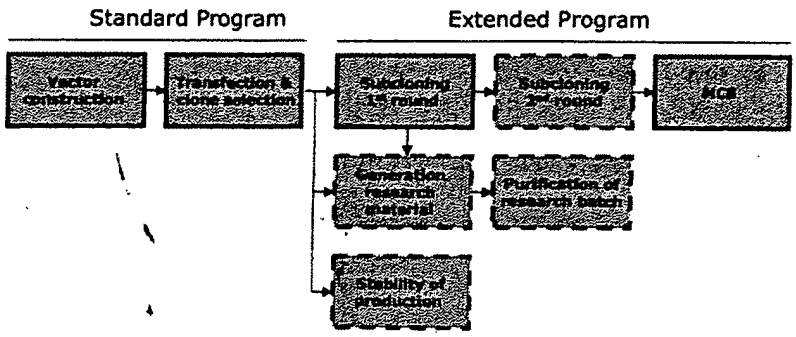

Fig. 3.3 Overview of the generation of PER.C6 clones producing high levels of monoclonal antibodies.

in total more than 15 times, mainly for IgG1 (κ and λ light chains, and for both allelic variants of IgG1 heavy chains [17]). In addition, IgM and IgA, as well as $F(ab)_2$ fragments have been expressed. A brief description of the selection of high-producing cell lines (summarized in Fig. 3.3), as well as adaptation to serum-free suspension conditions is presented, followed by a summary of the generic fed-batch and perfusion processes that have been developed for PER.C6 cells producing recombinant protein.

3.3.1 Vector Construction and Transfection

The first step in the production of monoclonal antibodies is generation of the expression construct. For antibody generation, the antibody construct depicted in Fig. 3.4 has mostly been used. Here, expression of both the light chain and the heavy chain genes is driven by a cytomegalovirus (CMV) promoter that has been modified to obtain high levels of gene expression in PER.C6 cells. Adherent PER.C6 cells, cultured in medium containing fetal bovine serum (FBS) are transfected with this construct. Cells that contain a stably integrated construct are selected using G418 (Geneticin). G418-resistant colonies are transferred to 96-well plates.

3.3.2 Primary and Secondary Screens

A total of 300–400 clones is isolated and transferred to 96-well plates and cultured in DMEM supplemented with 10% FBS. After 5–10 days, culture supernatants are sampled and screened for the presence of IgG either by Protein A HPLC or by ELISA. Production titers from two independent screening rounds are used to rank the transfectants, and the top 20–30 are selected and expanded for cryopreservation and further evaluation. Selection pressure is maintained until cryopreservation, after

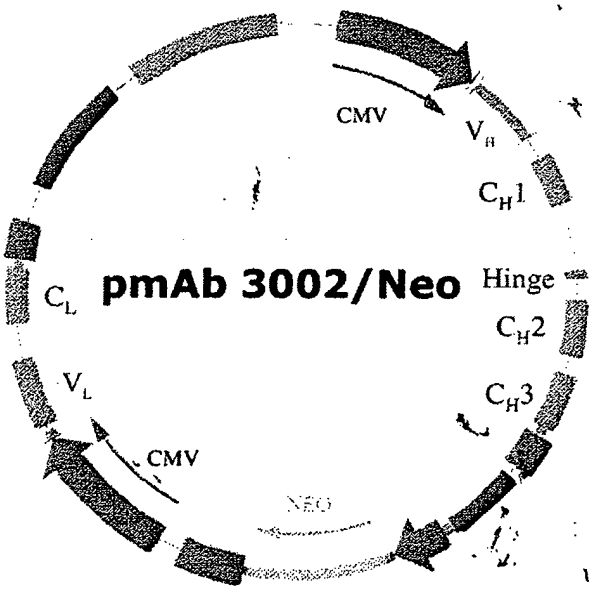

Fig. 3.4 Expression plasmid encoding IgG heavy and light chains used for transfection into PER.C6 cells.

which G418 is removed from the culture medium.

The highest ranked cell lines selected from the primary screens are then screened in 6-well plates using DMEM plus 10% FBS. Cells are seeded at 0.5×10^6 cells per well in duplicate and incubated for 4 days at 37 °C and 10% CO_2. Culture supernatants are then harvested and the IgG concentration determined by Protein A HPLC or ELISA. Final cell concentrations are measured and the data used to calculate cell-specific production rates. The cell lines with the highest cell specific production rate are selected for adaptation to serum-free conditions. Fig. 3.5 illustrates the results of a typical secondary screen performed for an internal clone generation program at Crucell. Fig. 3.5 A shows the final antibody concentration, and Fig. 3.5 B the cell-specific productivity. There is usually a good correlation between volumetric and cell-specific productivity; that is, cells with a high volumetric productivity show a correspondingly high specific productivity, and vice versa. Occasionally, a cell line with a high specific

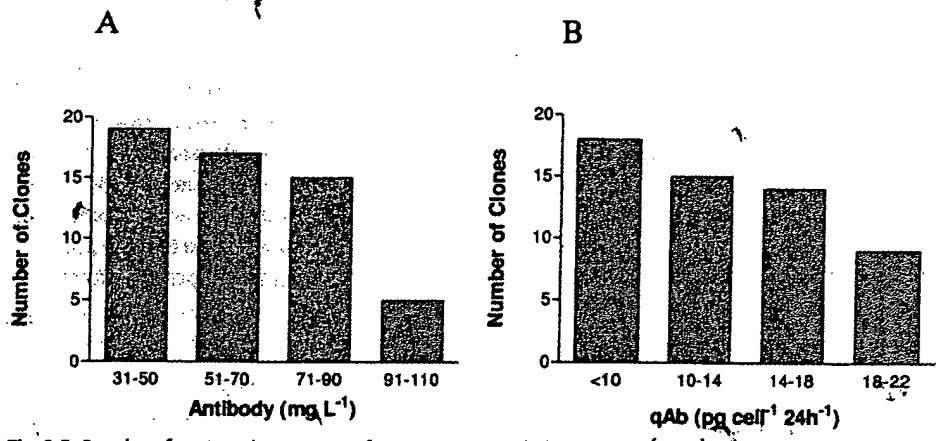

Fig. 3.5 Results of a secondary screen of 57 clones from a cell-line generation program conducted at Crucell. The screen was performed in 6-well plates using DMEM + 10% FBS. Cells were seeded at 0.5×10^6 mL^{-1} and the supernatant was harvested at day 4. (A) volumetric productivity; (B) specific productivity.

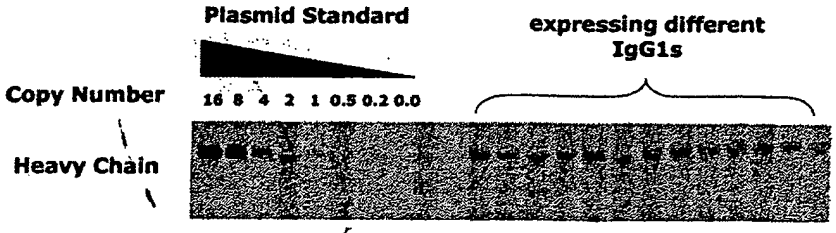

Fig. 3.6 Southern blot indicating the copy number of DNA encoding the light chain in different IgG expressing PER.C6 clones. Plasmid copies are measured to a standard comprising a known amount of plasmid DNA in a background of human chromosomal DNA.

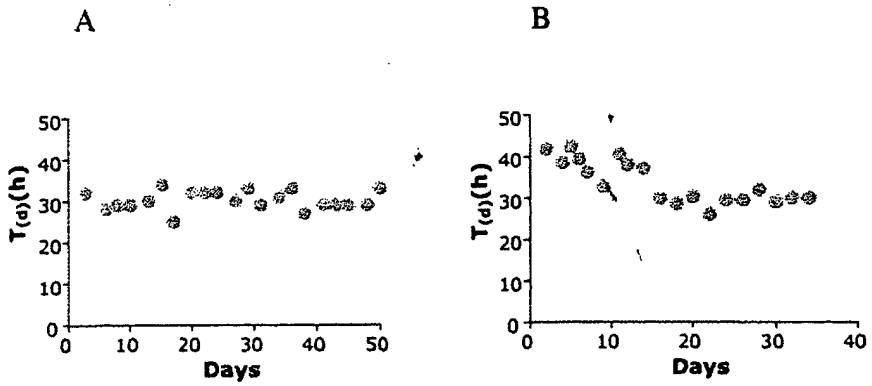

Fig. 3.7 Typical adaptation profiles for two different antibody-producing PER.C6 cell lines (A and B). Cells cultured in the presence of 10% FBS are transferred directly to serum-free media in Erlenmeyer shake flasks. Cells are passaged and population doubling time is calculated.

productivity will show a low volumetric production due to poor growth, or vice versa. In this example, 20 clones were selected for adaptation to serum-free conditions.

Selected PER.C6 cell lines possess low copy numbers, typically below 10 copies per cell as measured by Southern analysis (Fig. 3.6).

3.3.3 Selection Serum-free

Adherent cells are trypsinized and re-suspended directly in shake flasks containing serum-free medium. Cells are cultured every 2–4 days and incubated at 37 °C, 5% CO_2 and 100 r.p.m. The adaptation period for PER.C6 cell lines using this strategy is typically quite short, up to a maximum of 15–20 days. Fig. 3.7 shows two typically observed adaptation profiles for antibody-producing cell lines. Once adaptation is complete and a stable doubling time is observed, cell lines are evaluated in the desired production process, typically batch or fed-batch. Growth, production and metabolite profiles are characterized. The product is purified and analyzed by SDS-PAGE (no major contaminating and/or unexpected bands), IEF (conform previous produced material), HPLC-SEC (>90% monomer) and glycan analysis (correct galactosylation). A selection of one to three lead cell lines is then made based on process performance (growth rate, productivity, metabolic profile) and product quality. Fig. 3.8 shows the final antibody concentrations from batch culture for the 20 selected clones. In this example, seven clones showed yields above 0.5 g L^{-1} and were selected for further evaluation in fed-batch.

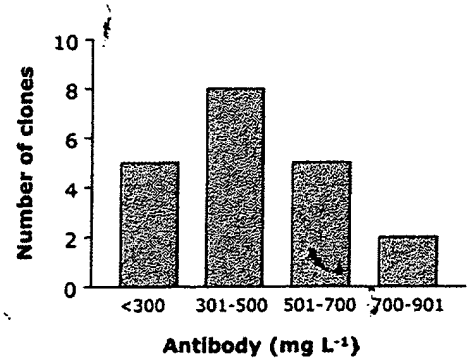

Fig. 3.8 Antibody yields from batch cultures for 20 antibody-producing cell lines adapted to serum-free media.

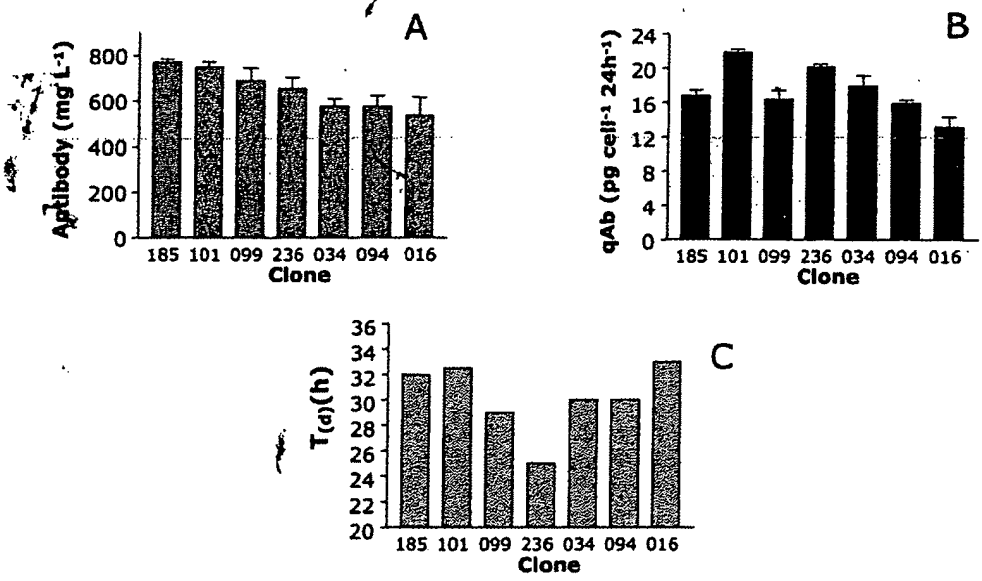

Fig. 3.9 Overview of the seven antibody-producing cell lines yielding more than 500 mg L^{-1} in batch culture (A), specific productivity ranging from 12 to 22 pg per cell per day (B), and population doubling time ranging from 24 to 34 hours (C).

Fig. 3.9A shows the final antibody concentration, Fig. 3.9B specific productivity, and Fig. 3.9C the doubling time for each selected cell line.

Once final selection has been made, cryopreserved cell stocks are prepared in serum-free medium.

3.3.4 Sub-cloning

Cell lines that are carried forward as potential production lines are sub-cloned. Cells are plated at an average of 0.3 cells per well in 96-well plates, and out-growing colonies screened, expanded, frozen, and tested as described for the initial clones.

3.3.5 Cell-line Generation Timelines

The aim of cell-line generation is rapidly to select high-yielding cell lines that perform optimally in the desired production process. The process from transfection to final selection of the lead clone (including evaluation in batch or fed-batch) takes 6–7 months (see Fig. 3.10). The aim is to move as quickly as possible to serum-free conditions and to make the final selection based on performance in one of the generic production processes, whether batch, fed-batch, or perfusion. The inclusion of such generic production processes in the selection program not only ensures that the lead cell line that will perform optimally in a production environment, but also reduces the amount of process development work required for each new cell line. An

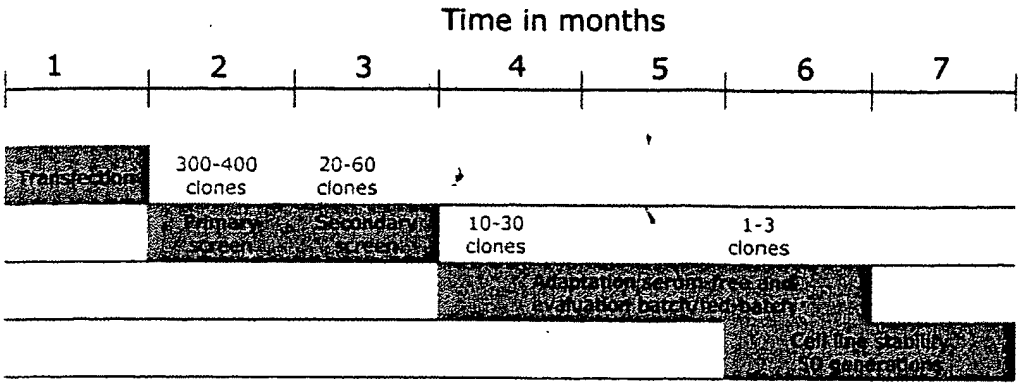

Fig. 3.10 Timelines for the generation of stable antibody-producing PER.C6 cell lines.

additional feature of the PER.C6 cell line that reduces the timeline for cell-line generation is the easy and rapid adaptation to serum-free conditions (see also Fig. 3.7), which typically requires less than 3 weeks by a direct adaptation in shake flask. Finally, expression in PER.C6 cells does not involve amplification of gene copy number, as for example in CHO DHFR$^-$ cell lines. As a result, recombinant PER.C6 cell lines can be relatively quickly selected and evaluated in the required production system, without the time normally needed for amplification.

3.4 Fed-batch Process Development

A generic fed-batch process has been developed for the production of monoclonal antibodies in PER.C6 cells. The process typically results in a 3- to 4-fold increase in antibody yields compared to the batch process, with yields of 1–3.5 g L^{-1} after 16–18 days. The feed strategy is based on the metabolic requirements of the PER.C6 cell line. Metabolic characterization of several antibody-producing cell lines identified nutrients and medium components which are important for the maintenance of growth and productivity. These were assembled in a nutrient concentrate consisting of glucose, phosphate and amino acids and a component concentrate consisting of vitamins, lipids, trace elements, salts and growth factors.

The feed strategy involves the addition of these nutrients based on cell-specific requirements in order to supply the nutrients only as required by the culture and to limit overflow metabolism or the build-up of nutrients or metabolites that may result in reduced process performance (antibody yields) and product quality [18–25].

In addition to a controlled feed strategy, physico-chemical process parameters have been optimized for process efficiency. For example, the growth rate of PER.C6 cells is optimal at pH 7.3 (Table 3.1). The cell-specific rates of nutrient utilization are highest at that pH (Table 3.1) however, with values for glucose, glutamine and phosphate for example up to two- or three-fold higher than at pH 6.9. This increased rate of nutrient utilization at pH 7.3 does not result in higher maximum cell yields or cell-specific productivities, and can thus be regarded as metabolically less efficient. It also has a significant influence on the

Table 3.1 Summary of metabolic and growth data for antibody-producing PER.C6 cell line (antibody A) in batch culture at different culture pH values.

	pH 6.9	pH 7.3	No low limit pH control
qGlc	0.6	1.6	0.7
qGln	0.17	0.32	0.18
qPhos	0.05	0.12	0.07
Nv (max.)	9.8	10.2	10.5
Avge T(d) day 1–4	38	28	31
qAb	14–16	14–16	14–16

design and efficiency of a fed-batch process, as a feed strategy at pH 7.3 would involve the addition of two to three times the nutrient concentrations as for a process at pH 6.9. This would give increased osmolality and result in reduced process performance. The problem with operating a process at pH 6.9 is the sub-optimal growth rate compared with pH 7.3, which results in a longer process. This was overcome by controlling the starting pH of cultures to 7.3, but then operating without a low limit pH control. In PER.C6 cell cultures this resulted in a pH "drift" down to approximately 6.9 during growth, which led to a culture that showed optimal growth rates and nutrient utilization profiles. Operating the process with such a pH drift also reduces lactate accumulation. PER.C6 cells possess a lactate transport system that is a proton symport system and thus is dependent on a low extracellu-

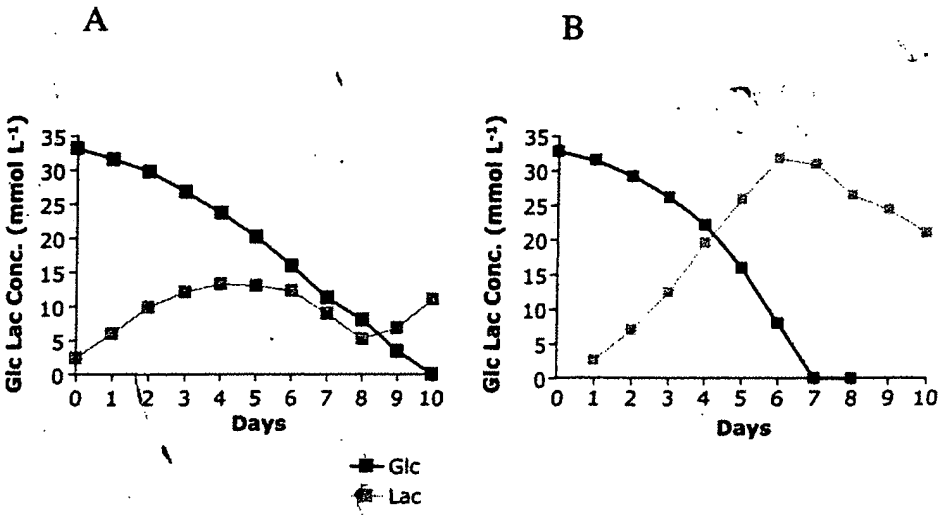

Fig. 3.11 Glucose utilization and lactate production profiles for: (A) a batch culture operated with no low limit pH control (initial culture pH 7.3); and (B) a batch culture operated with pH control at 7.3.

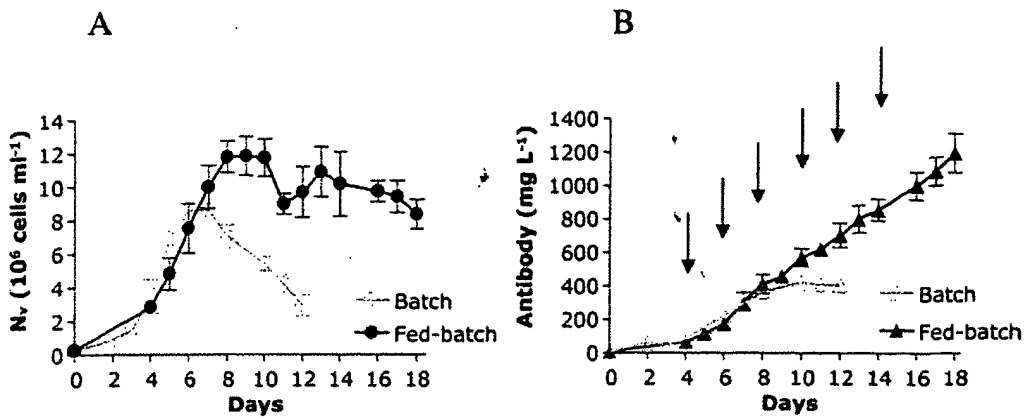

Fig. 3.12 (A) Cell and (B) antibody profiles for batch (open symbols) and fed-batch cultures for a PER.C6 cell-line expressing antibody A. The data represent an average of eight 2-L bioreactor runs.

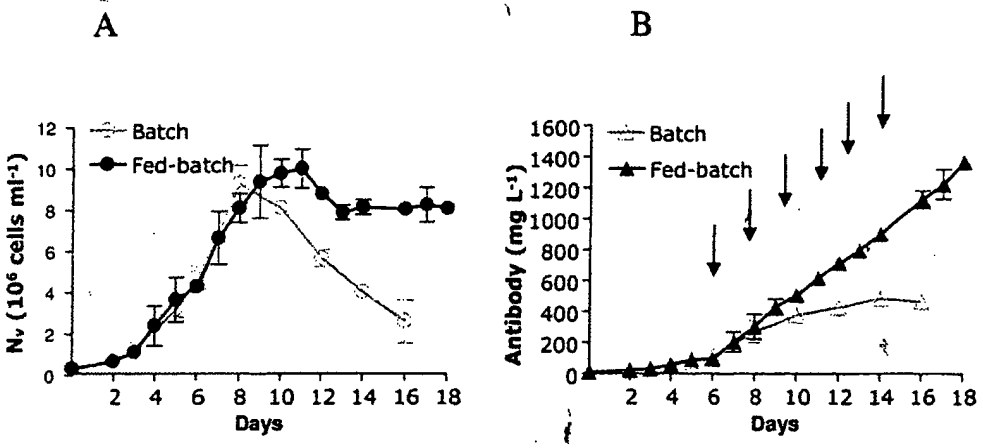

Fig. 3.13 (A) Cell and (B) antibody profiles for batch (open symbols) and fed-batch cultures for a PER.C6 cell-line expressing antibody B. The data represent an average of three 2-L bioreactor runs.

lar pH. When cultures are operated with no low limit pH control therefore, there is a period of lactate release and the pH decreases. As this occurs, lactate transport starts and extracellular lactate concentrations plateau and begin to decrease. However, if pH is maintained at 7.3, no lactate transport is observed and lactate accumulates in the culture (Fig. 3.11).

A typical feed strategy involves the addition of four to six bolus feed additions at regular intervals during a 16- to 18-day process. Similar growth and production profiles are observed for all antibody-pro-

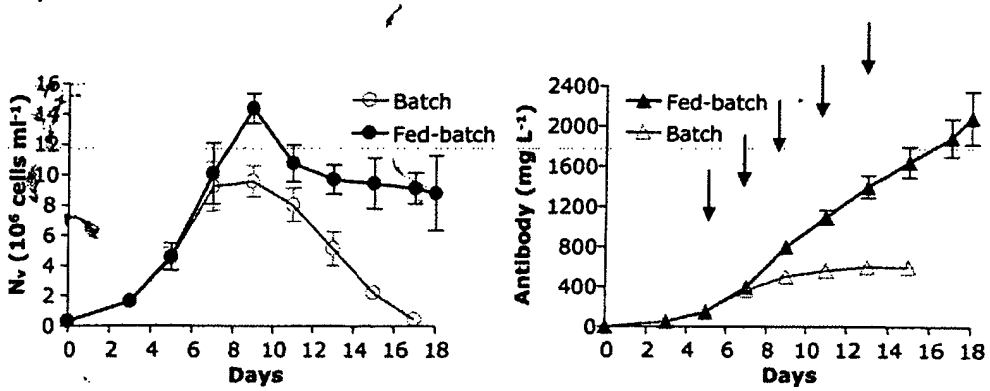

Fig. 3.14 (A) Cell and (B) antibody profiles for batch (open symbols) and fed-batch cultures for a PER.C6 cell-line expressing antibody C. The data represent an average of 18 shake flask runs.

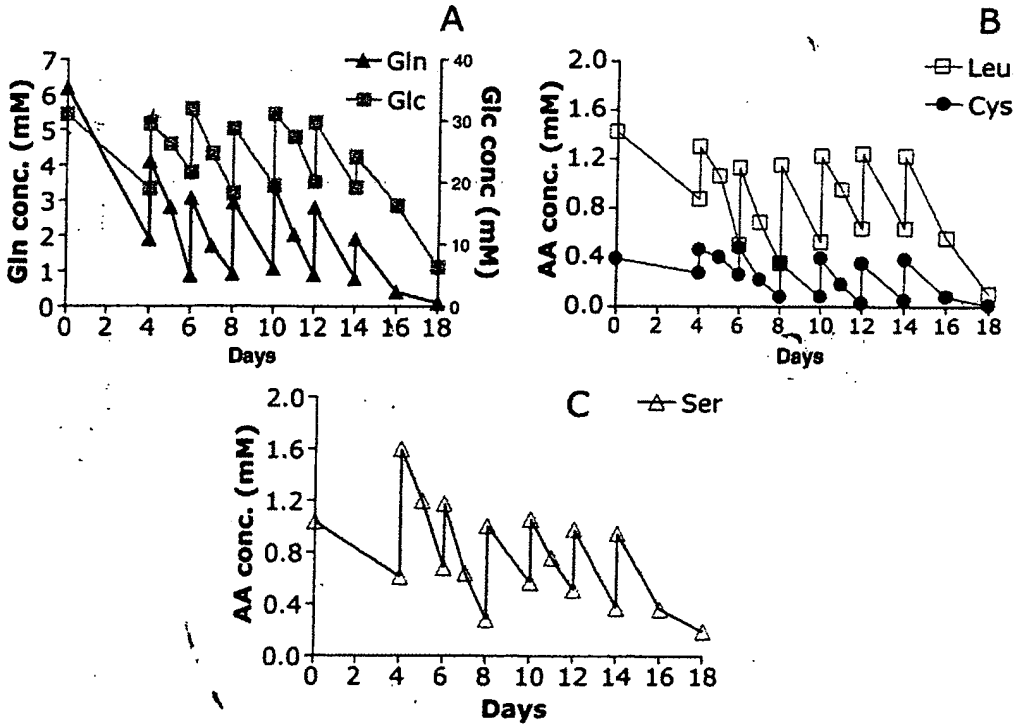

Fig. 3.15 Nutrient profile during a fed-batch culture of a PER.C6 cell-line expressing antibody A. The data show the concentration of (A) glutamine (closed triangles), glucose (closed squares); (B) leucine (open squares), cystine (closed circles); and (C) serine (open triangles).

Table 3.2 Summary of results obtained with four PER.C6 cell lines expressing different IgG1

	Batch [g L^{-1}]	Fed-batch [g L^{-1}]	Process length [days]	qAb [pg/cell/day]
Antibody 1	0.4	1.3	18	12–15
Antibody 2	0.5	1.4	18	10–12
Antibody 3	0.6	2.1	18	15–18
Antibody 4	0.6	1.8	16	16–19

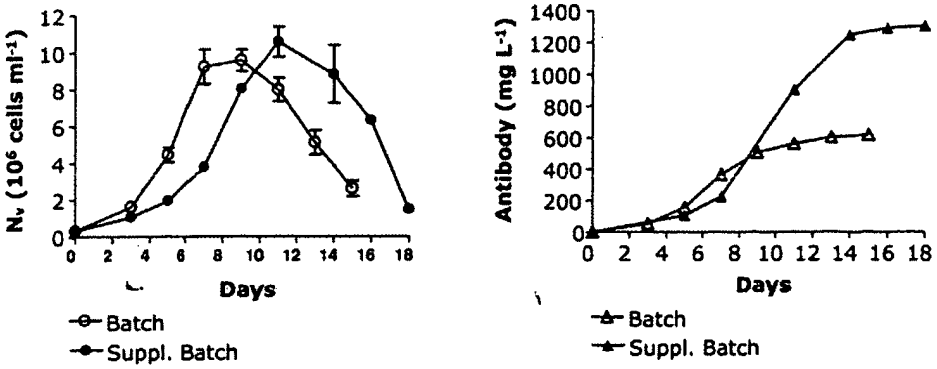

Fig. 3.16 (A) Cell and (B) antibody profiles for a PER.C6 cell-line expressing antibody C in batch and supplemented batch cultures. Supplementation of the batch was made prior to inoculation, with 50% of the feed added to a typical fed-batch culture. The data represent the average of four shake flasks.

ducing cell lines that have been evaluated in the fed-batch process. Figs. 3.12, 3.13 and 3.14 show the growth and production profiles of three different antibody-producing cell lines (cell lines A, B, and C, respectively) in batch and fed-batch. Table 3.2 shows a summary of the same three cell lines (A, B, and C) and a fourth (cell line D) evaluated in batch and fed-batch, and includes the average specific productivity. Fig. 3.15 illustrates selected nutrient profiles during the fed-batch for one of the cell lines (cell line A), showing that concentrations of the added nutrients remain stable during the fed-batch and that the feed is accurately matched to the metabolic requirements.

3.4.1 Supplemented Batch Process

Metabolic data from the fed-batch development was used to develop a supplemented batch process involving the addition of up to three of the feeds added in the fed-batch process, to the culture medium prior to inoculation of the cells. Final antibody yields are not as high as for the fed-batch process, typically an increase of 2-fold over batch yields compared to 3- to 4-fold increases for the fed-batch. However, the process offers a relatively simple way of obtaining increased antibody yields. Fig. 3.16 shows a supplemented batch culture for clone C where the final antibody concentration reached 1.3 g L^{-1}.

3.5 Operation of PER.C6 Cells in Continuous Perfusion

Perfusion presents a number of advantages over other modes of cultivation, such as increased volumetric productivity and rapid removal of easily inactivated products from the culture environment [26]. These homogeneous perfusion systems can be operated under conditions of total cell retention, or with the removal of part of the biomass through culture bleeding [27]. Total cell retention facilitates kinetics studies and, as demonstrated in other studies [28], prevents unnecessary cell division, allowing for cells to produce product at higher rates [29].

A perfusion process has been developed for the production of monoclonal antibodies in PER.C6 cells. The process typically results in a more than 30-fold increase in antibody yield compared to the batch process, and a 10-fold increase compared to the fed-batch process. As for the development of the fed-batch process, the perfusion strategy is based on the metabolic and physico-chemical requirements of the PER.C6 cell line.

3.5.1 Initial Assessment

The first feature investigated was the impact of cell perfusion rate (CSPR) on cell culture performance using a spinfilter as the cell retention device. Fig. 3.17 shows the viable cell concentration of a PER.C6 cell line expressing antibody A operated under the same conditions with two different CSPR implemented at the 7-L scale.

It was observed that a doubling of the CSPR resulted in a 65% increase in the upper cell density. The fact that such an increase was achieved suggested that significant improvements in cell density (and related productivity) could be obtained by modification of the CSPR. By day 18, both of these cultures were prematurely ended due to clogging of the spinfilter. Upon inspection of the spinfilter material, a cake of cells was observed to have gathered, which suggested that the high cell densities achieved with this culture are not compatible with a standard spinfilter operation as a cellular retention device.

A complete metabolic characterization of these cultures was performed (data not

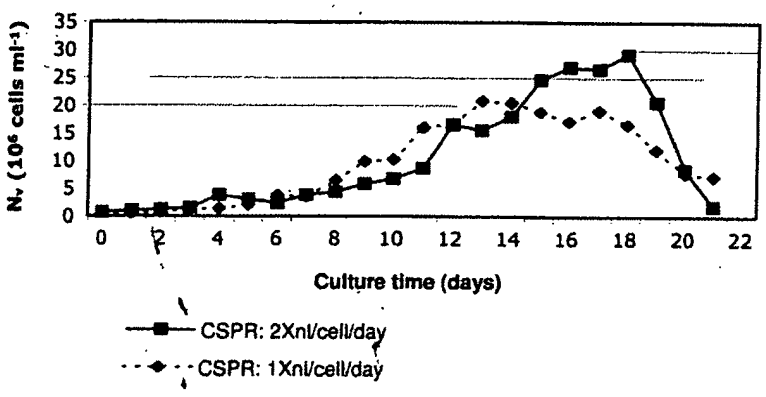

Fig. 3.17 Cell concentration ($\times 10^6$ cells mL^{-1}) versus culture time (days) for PER.C6 cell line expressing antibody A operated at two different cell perfusion rates (CSPR).

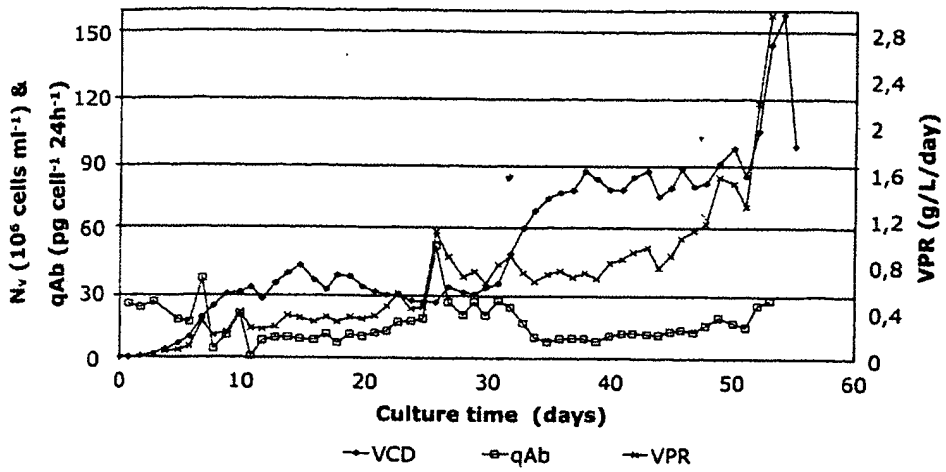

Fig. 3.18 Viable cell density (VCD; $\times 10^6$ cells mL^{-1}), cell-specific production rate (qAb; pg per cell per day) and volumetric production rate (VPR; g L^{-1} per day) versus culture time (days) for PER.C6 cell line expressing antibody A in a continuous perfusion.

shown); post analysis of a modified perfusion strategy was assessed.

3.5.2 Stage 1 Development

Fig. 3.18 details the cell profile achieved during round 1 development of a continuous perfusion process using a PER.C6 cell line expressing antibody A. The viable cell concentration reached approximately 30×10^6 mL^{-1} by day 12 and remained constant at this level for a further 20 days. At this point, a series of actions were taken which resulted in the cell concentration increasing to approximately 80×10^6 mL^{-1} by day 38. Conditions were then kept constant for a 14-day period during which a constant cell concentration was maintained. On day 52, the same process change as operated at day 32 was implemented which led to a further increase in the viable cell concentration to 155×10^6 mL^{-1} by day 53. During the next 24 hours, the cell retention device became unusable due to the high cell concentrations, and this led to termination of the run. The cell viability throughout the process was greater than 95%.

The specific production profile (q_{Ab}) of the cells was stable for the majority of the process, with an average of 15 pg per cell per day. Higher values at the end of the process were due to cell retention device failure. High volumetric production rates (VPR) were achieved during this process, with an average VPR of 0.76 g L^{-1} per day calculated over the entire process, 0.9 g L^{-1} per day at approximately 80×10^6 mL^{-1} and an upper VPR of 3 g L^{-1} per day achieved at 150×10^6 mL^{-1}.

3.5.3 Stage 2 Development

Round 2 development consisted of identifying the critical features necessary to achieve the extreme cell densities. Fig. 3.19 shows the viable cell concentration ($\times 10^6$ cells

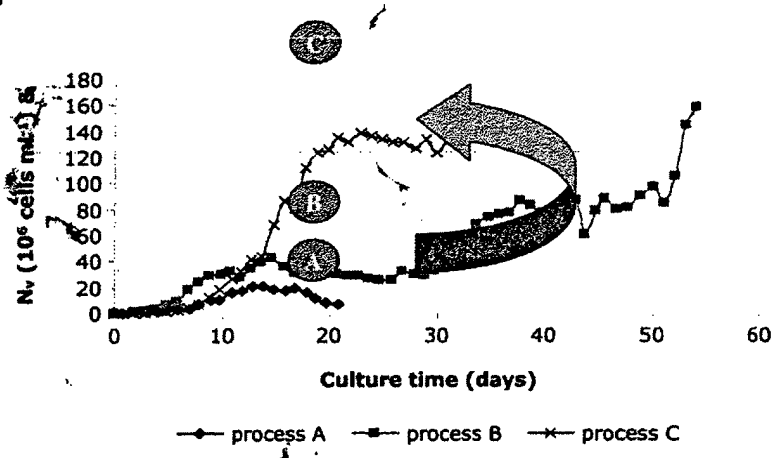

Fig. 3.19 Viable cell concentration ($\times 10^6$ cells mL^{-1}) versus culture time (days) of the optimized modified process compared to the initial experiments.

mL^{-1}) versus culture time (days) achieved using the round 2 modified process.

The progression gained from each of the stages can be visualized in Fig. 3.19. It should be noted that biphasic growth profile observed in process B is reduced to a monophasic growth profile by process C. In addition, there is significant reduction in the process time required to achieve cell concentrations of 100×10^6 cell mL^{-1}. Process C requires approximately 16 days to achieve cell concentrations of 100×10^6 cells mL^{-1}, while process B required approximately 40 days to reach 80×10^6 cells mL^{-1}.

In summary, and to the present authors' best knowledge, the cell concentrations achieved with the PER.C6 cell line (see Fig. 3.20) are the highest reported value to date for a mammalian cell line. These results propel the overall productivity for a PER.C6 cell line to the highest reported values for an antibody-producing process.

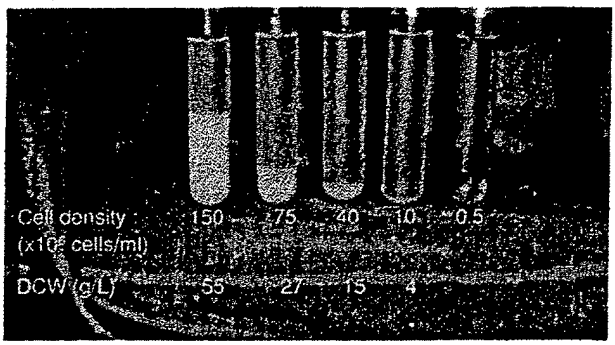

Fig. 3.20 PER.C6 cell concentration with corresponding dry cell weights (DCW) (g L^{-1}) obtained during a perfusion mode of operation.

3.6 Characterization of Antibodies Produced by PER.C6 Cells

One of the cornerstones in the development of biopharmaceuticals is the availability of reliable, sensitive and accurate analytical methods to characterize recombinant products [30]. Typically, these methods are divided into several categories: product quantity, identity, purity, and potency testing [31]. Product characterization methods can be general for any recombinant protein or specific to a particular drug substance. In addition, new analytical technologies and modifications to existing technologies should be used whenever the methods can add valuable data to process development and lead to a better understanding of the consequences of process changes to ensure the safety and efficacy of the product in patients. These methods should be set up during the process development phase of a biopharmaceutical product. This section summarizes the analytical results obtained in parallel with the development of processes for the production of monoclonal antibodies in the PER.C6 cell line. General analytical methods that are used to release materials from production such as sterility testing or in-process testing such as process-related impurities and indicators are beyond the scope of the present review, or are shown as part of process development.

Quantity is an important product characteristic, and is usually the first method to be implemented during process development. There are a number of physicochemical tests available to measure the antibody content of cell culture samples, including ELISA, UVA_{280} and affinity chromatography [32]. Typically, ELISA methods to assay IgG content are very sensitive and used to quantify low levels of crude biological products such as those seen in clone selection or in microscale processes; a large number of these methods are commercially available [33]. Several ELISA methods have already been successfully implemented to quantify antibody titers produced in the PER.C6 cell line.

Our method of choice to quantify recombinant antibodies produced in PER.C6 cells is that of analytical protein A chromatography (APAC). This method is automated, rapid (total run time of 3.8 min), precise (standard deviation < 5%), and sensitive (quantitation limit 25 $\mu g\ mL^{-1}$), and generally outperforms ELISA methods, except for sensitivity.

There are a number of features of monoclonal antibodies that are identified as critical quality attributes. Much of the discussion between biotechnology companies and regulatory agencies centers on the choice of the appropriate methods to demonstrate product consistency for lot release. In the present project, since the novelty of the technology is the use of human cells in the production of recombinant antibodies, the focus of the characterization studies is general characteristics such as glycosylation and protein profiles [34]. In particular, the exhibition of the predictive nature and consistency of glycan and protein heterogeneity is important in order to validate the PER.C6 cell line as an attractive expression system for the manufacture of antibodies.

It was necessary to purify the monoclonal antibodies that were produced in PER.C6 cells in order to examine the structural features of these products. To accomplish this, a two-step purification method was executed whereby cell culture material was loaded onto a semi-preparative protein A column and the antibodies were eluted at low pH [35]. The eluted antibodies were injected directly on a desalting column and then concentrated in order to prepare the

samples for characterization analyses. The purification of the antibodies produced in PER.C6 cells was straightforward, and recoveries exceeded 75%. The samples were very pure after the affinity step, as shown by SDS–PAGE analysis and high-performance size exclusion chromatography (HP-SEC). The level of higher molecular weight impurities in the samples was very low (typically <1.5%). Once purified, samples were subjected to a number of identity assays including gel and capillary electrophoresis and glycosylation analysis.

3.6.1 SDS-PAGE

The conventional slab gel technique SDS-PAGE is an important technique for the routine identity and purity analysis of biotechnology products [36]. A typical SDS-PAGE analysis, run under reducing conditions with Coomassie blue staining, is shown in Fig. 3.21A. The heavy and light chains of the purified antibody samples are clearly seen in all of the loaded samples. Molecular weight (MW) markers that encompass the bands of interest are also loaded onto the gel. No differences have been detected between cultures run in batch, fed-batch or perfusion modes.

3.6.2 Isoelectric Focusing

Isoelectric focusing (IEF) is another routine slab gel technique for the identification of biotechnology products [37]. The isoelectric points (pI) of the produced protein and its variants can be monitored. The presence of several charge variants is a common feature of recombinant antibodies and can be the result of, for example, deamidation or differences in processing of C-terminal lysine residues [38, 39]. The consistency of the charge heterogeneity can be monitored by using IEF. A typical IEF analysis is shown in Fig. 3.21B. The pI values of the protein isoforms can be identified for all of the samples. The IEF patterns for antibodies produced in batch, fed-batch and perfusion modes with PER.C6 cells are very similar.

Unlike its conventional slab gel counterpart, capillary isoelectric focusing (cIEF) is automated, precise and quantitative [40]. A typical cIEF profile of a purified human IgG1 that was produced in PER.C6® cells is shown in Fig. 3.22. In this case, five isoforms can be identified and quantified. The major isoform has a pI value of 8, which is typical for human IgG1.

During the process development of monoclonal antibodies production in PER.C6

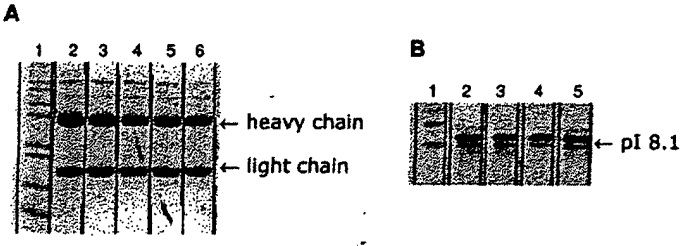

Fig. 3.21 (A) SDS-PAGE analysis under reducing conditions of human IgG1 produced in PER.C6 cells. Lane 1: MW markers; lanes 2 and 4: IgG1 produced in batch process; lanes 3, 5, and 6: IgG1 produced in fed-batch process. (B) IEF gel of human IgG1 produced in PER.C6 cells. Lane 1: pI markers; lanes 2 and 4: IgG1 produced in fed-batch process; lanes 3 and 5: IgG1 produced in batch process.

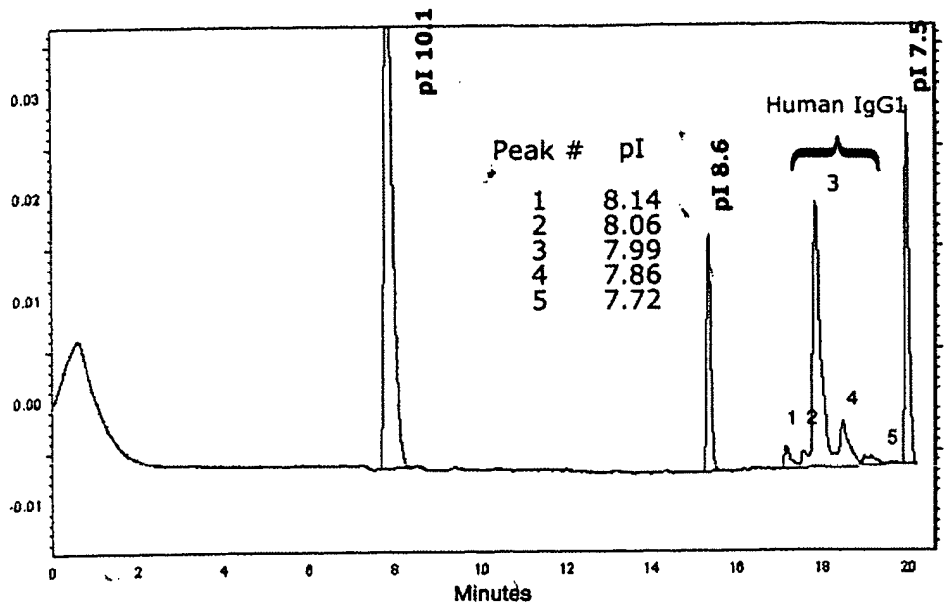

Fig. 3.22 A typical capillary isoelectric focusing profile of purified human IgG1 from PER.C6 cells. The peaks at pI values of 10.1, 8.6, and 7.5 are pI markers.

Table 3.3 Typical pI values of the five isoforms for purified human IgG1 samples produced in batch, fed-batch, and perfusion modes.

Sample	Peak 1 [%]	Peak 2 [%]	Peak 3 [%]	Peak 4 [%]	Peak 5 [%]
Batch – end	3	6	59	24	8
Fed-batch – mid	N/D	4	66	21	9
Fed-batch – end	4	7	67	17	5
Perfusion – early	N/D	4	87	6	3
Perfusion – mid	N/D	N/D	87	10	3
Perfusion – late	N/D	N/D	87	10	3

Relative peak areas were obtained by the integration of the major isoforms in the cIEF electropherograms of the samples.
N/D = not detected.

cells, several in-process samples were taken from the production vessel, purified, and subjected to cIEF analysis. Typical results of these analyses are shown in Table 3.3. Excellent quality control is seen in both fed-batch and perfusion modes, since the cIEF profiles of samples taken at the mid and end-points of the runs were very similar. Inter-assay variation is ± 3% for each isoform of the same sample. Minor differences in cIEF profiles can be seen for samples taken from the three modes of manufacturing. Presum-

ably, the charge heterogeneity is due to differences in deamidation levels caused by the different production conditions.

3.6.3 Glycan Analyses

All eukaryotic recombinant expression systems produce therapeutic proteins that are glycosylated in their native state for *in vivo* activity [41]. The expressed glycoprotein contains glycosylation variants, called glycoforms, which are the subject of a number of recent reviews [42–45]. They are also discussed in detail in this book (Part IV, Chapter 1, 2, 7; and Part VI, Chapter 2). Since glycosylation of biological molecules is achieved through a complex, post-translational pathway involving several enzymes, carbohydrate structures are very sensitive to even subtle differences in the environment in which they are formed. The main factors that influence the oligosaccharide profile of glycoproteins are cell line, cell culture medium, bioreactor parameters, harvest time, and manufacturing site changes.

In the present case, all IgG1 molecules contain a conserved N-glycosylation site at asparagine 297 in the constant region of the molecule. In human serum, the major glycoforms of IgG1 contain a biantennary structure with a core fucose [46]. The three most common glycans found in IgG1 molecules, G0, G1, and G2, are shown in Fig. 3.23.

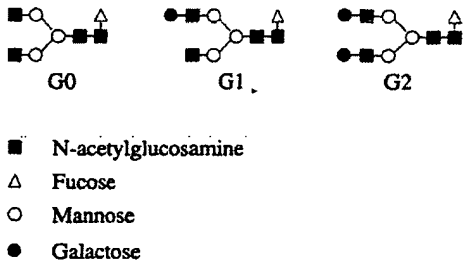

Fig. 3.23 Structures of the three most common glycans found in human IgG1 molecules, G0, G1, and G2.

Purified antibody samples from development runs were subjected to PNGase F treatment to release the glycans, and these were analyzed by either MALDI-MS or HPAEC-PAD to determine the glycan structures.

IgG1 produced by PER.C6® cells in batch culture show a similar galactosylation profile to human serum IgG [34], with approximately 30% G0, 50% G1, and 20% G2 (Fig. 3.24). This can be compared to CHO-produced antibodies, which are typically

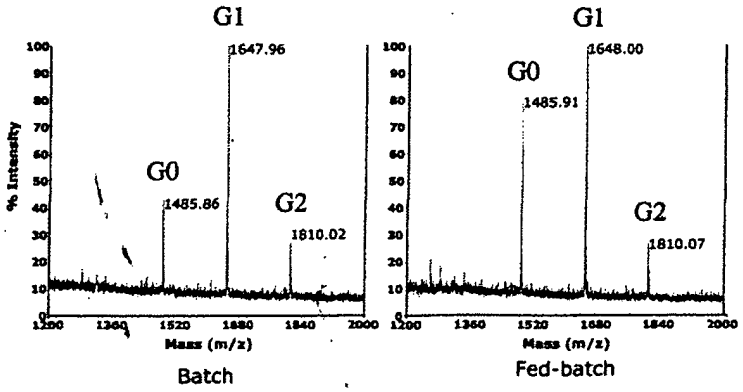

Fig. 3.24 MALDI-TOF traces of glycans of antibody B produced in batch and fed-batch.

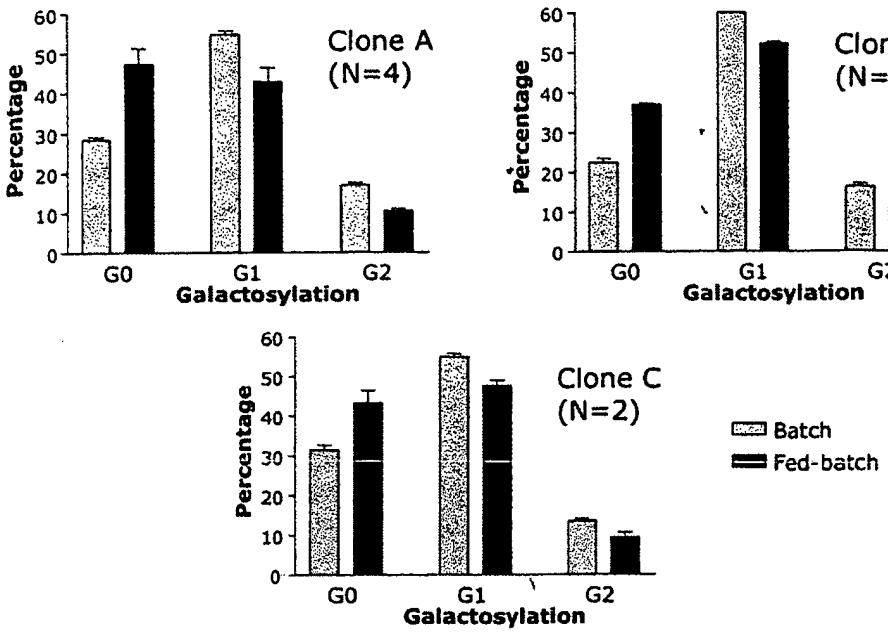

Fig. 3.25 Galactosylation profile of three different monoclonal antibodies produced in batch and fed-batch processes. The profiles were made by MALDI-TOF analyses.

produced predominantly in the G0 form. For example, Hills et al. [47] reported a galactosylation profile for an antibody produced in NS0 and CHO cells of approximately 60% G0, 35% G1, and 5% G2 for the NS0 cell line, and 63% G0, 33% G1, and 4% G2 for the CHO cell line. A small decrease in galactosylation is typically observed in the fed-batch-produced antibody, with the percentage of G0 glycoforms increased from 30 to 45%, and the percentage of G1 and G2 glycoforms decreased from 50% and 20% to 40% and 15%, respectively. Fig. 3.25 shows the galactosylation profile of three different antibodies produced in batch and fed-batch culture from three different PER.C6 cell lines. This reduction in galactosylation is likely due to the different culture conditions between the two processes, such as an increase in process length and in final

Table 3.4 Glycan profile by HPAEC-PAD chromatography of human IgG1 produced in PER.C6 cells in the three manufacturing modes. The normal ranges of the three major isoforms are presented

Sample	G0 [%]	G1 [%]	G2 [%]
Batch	18–27	55–59	15–25
Fed-batch	18–27	55–59	15–25
Perfusion	11–20	53–57	20–33

ammonia concentrations (ammonia concentrations typically reach 10–12 mM by the end of the fed-batch compared to 3–4 mM for the batch process). The effects of ammonia on glycosylation have been well reported [48–50], and appear to be due to the activity of ammonia as a weak base, increasing the pH of the lumen of the Golgi body. In general, the distribution of the G0, G1, and G2 isoforms varies only slightly between cell culture runs. Inter-assay variation is ±0.5% for each isoform of the same sample. Only slight differences have been observed between the cell lines analyzed to date in either batch and fed-batch modes. Little or none bisecting *N*-acetylglucosamine or sialic acid is present, and no evidence of structures that may be immunogenic in humans, such as high-mannose or hybrid structures has been detected. These structures have been reported for glycoproteins produced in non-human cell lines, such as NS0 (a mouse myeloma cell line) [51]. An interesting observation is that the recombinant antibody is generally more galactosylated in perfusion mode (Table 3.4), where the cell counts are the highest ($\sim 10^8$ cells mL^{-1}).

3.6.4 Peptide Mapping

Peptide mapping using liquid chromatography (LC) coupled with either UV detection or mass spectrometry (MS) is a powerful technique to study the primary structure of the antibody, and to further investigate post-translational or chemical modifications. The peptide map is a chromatographic finger-print which is obtained after reduction/alkylation and subsequent proteolytic digestion of the antibody (Fig. 3.26). Thus obtained UV-patterns are used routinely to screen for structural integrity after process changes or for quality control of production lots [52]. LC-MS is applied to confirm the amino acid sequence and to detect and identify modifications.

The primary sequences of PER.C6 cell-derived IgG1 and IgG4 antibodies were confirmed in LC-MS peptide maps, and no changes compared to the sequence expected from DNA-transcription were detected. The presence of the typical glycan structures (see Fig. 3.23) could be confirmed, and it was demonstrated that non-glycosylated heavy chains were not present. Modifications of the N- and C-terminus of the heavy chains were observed in both IgG1 and IgG4 antibodies. In all cases, the N-terminal glutamine residue was converted by cycliza-

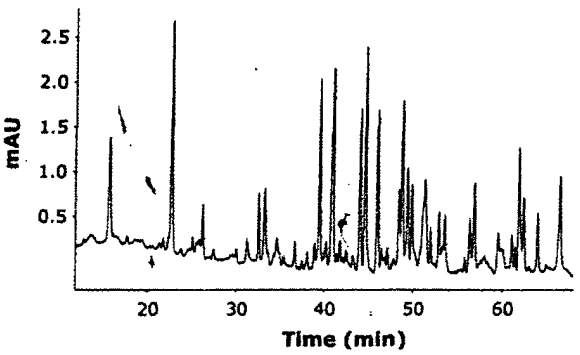

Fig. 3.26 Peptide map of a PER.C6-derived IgG1 using liquid chromatography coupled with UV detection.

tion into a pyroglutamate residue, which is a common chemical modification of antibodies. The C-terminal modification is caused by removal of the C-terminal lysine residue from the heavy chain. This is due to the activity of carboxypeptidases, and is a frequently observed characteristic of proteins produced in mammalian cell culture [53]. Both the pyroglutamate conversion and removal of the lysine residue can contribute to charge heterogeneity of the product. However, in the described antibodies the conversions were 100%, resulting in homogeneous N- and C-termini. The charge heterogeneity observed in the IEF-analyses is most likely caused by deamidation of asparagine residues. The exact deamidation sites and the percentage of deamidated forms could be established with LC-MS.

3.6.5 Summary Biochemical Analyses

A number of analytical methods have been used to characterize monoclonal antibodies produced in PER.C6 cells. It has been shown that the antibodies can be quantified and purified easily, and several quality attributes can be maintained in batch, fed-batch, and perfusion production modes. The protein and carbohydrate structures of the antibodies are completely human in nature, and excellent control of these attributes during process development has been observed.

In line with this, the bio-activity of PER.C6 cell-produced monoclonal antibodies was shown to be equal or better than antibodies produced in CHO or murine cell lines (results not shown).

3.7 Conclusion

The PER.C6 cell line was generated from retina-derived primary human cells, which were immortalized by insertion of the adenovirus E1 gene. In comparison to CHO and NS0 cells, for example, the PER.C6 cell line has been used for only a few years in protein production. Nonetheless, it is already an attractive expression platform that exhibits many favorable characteristics for the production of IgG and other proteins. These include the very high cell numbers and hence high yields that are obtained, the rapid generation of high-expressing clones that match a generic and robust fed-batch process, and the high quality of the antibodies produced. These issues are discussed below.

3.7.1 Rapid Generation of High-producing Clones

The transfection of PER.C6 cells with expression plasmids is very efficient, as is the subsequent generation of stable cell lines (an overview of the process is shown in Fig. 3.3). Importantly, high expression levels of recombinant antibodies are observed in the absence of gene amplification, giving a considerable time advantage over cell lines which require amplification for efficient protein expression. High-expressing PER.C6 cell lines contain between two and 10 copies of antibody genes per genome, compared to hundreds of copies in amplified cell lines. A high gene copy number is associated with instability of expression over time. In contrast, expression from PER.C6 cell lines producing antibodies is very stable over several months.

Because the amplification of gene copy number is not required in PER.C6 cells, and thanks to the rapid and easy adaptation

to serum-free and animal component-free media, the timelines for cell line generation are minimal – 6–7 months from transfection to final lead clone selection for antibody-expressing PER.C6 cell lines. These cell lines are selected based on their performance in generic batch or fed-batch processes, thereby ensuring the selection of cell lines that perform optimally in the desired production process and reducing the investment in process development required for the rapid generation of material for pre-clinical and Phase I clinical studies.

3.7.2 High Numbers of Viable Cells

The generic production processes are based on the metabolic and physico-chemical requirements of PER.C6 cells. The specific productivity of antibody-producing cell lines analyzed to date ranges typically from 10 to 20 pg per cell per day. This does not compare with highly amplified CHO cell lines, for example, but it does result in high batch (0.4–0.8 g L^{-1}), fed-batch (1.3–2.2 g L^{-1}) and perfusion (up to 1 g L^{-1} per day) due to the high cell numbers obtained in these processes, which is on a par with levels obtained in industry with CHO and other cell lines. The reason for the high viable cell numbers may be related to the high-level expression of E1B proteins, which are known to be anti-apoptotic.

3.7.3 Consistent Product Quality of the Antibodies

Antibodies produced on PER.C6 cells show consistent product quality, as measured by IEF, SDS–PAGE and glycan analysis in batch, fed-batch, and perfusion processes. Typical glycan profiles for antibodies produced in PER.C6 cells are similar to human serum IgG, with a ratio of G0:G1:G2 of approximately 30%:55%:15% (see Fig. 3.25). Galactosylation is slightly reduced in the fed-batch, with an increase in G0 to 40–45% and a decrease in G1 and G2 to 45–50% and 10%, respectively. This decrease is probably due to the influence of ammonia, which reaches a higher concentration in the fed-batch.

To date, the profiles obtained are similar for all antibodies produced in PER.C6 cells, independent of the production levels obtained. Thus, the outcome of a production process becomes highly predictable.

By contrast, the majority of CHO-derived IgGs contain low levels of galactose, which may diminish the antibody's ability to initiate effector functions. In addition, in CHO cells sialic acid is added only via an α(2-3) linkage, whereas the sialic acid linkage in human serum may be α(2-6) or α(2-3). NS0 cells exhibit similar characteristics, but may also add an extra galactose to an existing terminal galactose via an α(1-3) linkage. Humans lack the enzyme that adds this structure, and such a Gal α(1-3) structure is highly immunogenic in humans: indeed, it is estimated that 1% of circulating Ig is directed against this moiety. Glycans with high-mannose structures and hybrid structures have also been observed on IgGs produced in CHO and NS0 cells. However, no such structures have been identified in antibodies produced in PER.C6 cells.

3.7.4 Future Prospects

As yet, the PER.C6 cell line has been used for protein production for only a relatively short period of time, but the high cell densities obtained, the generic fed-batch process and consistent product indicate that the cell line has vast potential. Unequalled high cell densities (> 150 · 10^6 cells mL^{-1}) ob-

tained in continuous perfusion can be used to manufacture very large amounts of proteins in a small-scale reactor. In addition, this procedure may be used to produce unstable proteins in an efficient manner.

The process also demonstrates that the maximum cell densities obtained in fed-batch can be improved significantly, and hence further increase the yields. The ultimate aim is a production platform on which large quantities of high-quality protein are produced at low cost, thereby allowing more people to benefit from effective, but expensive, biopharmaceuticals.

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